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MICROBIAL LOAD MONITOR

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1.0 SUMMARY

Work under Contract NAS 9-10516 is successfully concluded. We successfully performed all tasks outlined in the Statement of Work. Condensations of salient features follow:

a) We demonstrated our total MLM system to function as a diagnostic instrument capable of accepting human clinical specimens. Medically important microorganism detection is rapid and sensitivity is excellent.

b) There are three basic MLM system parts:

1) the MLM detection instrument

2) the MLM cartridge which contains the diluent (water) and vacuum Manifold for liquid transfer and filling, and

3) the filter cassette which serially dilutes the inoculum, and provides selective nutrients and growth chambers for MLM viewing.

c) We conducted tests with 143 clinical specimens obtained from a local hospital. In this double blind study, we compared results obtained from standard clinically analyzed specimens with tested MLM medium results obtained with the same specimen. MLM media selectivity was outstanding. In a few cases our MLM media detected pathogens' presence which were standard test missed; in a few other cases the MLM failed to detect Proteus specimens standard test detected. MLM media false positives never exceeded 3.3% in the worst case.

d) We tested over 200 clinical specimens freshly isolated obtained from a local hospital in MLM designed culture media. "Wild strains" selectivity proved as effective as for laboratory strains. We tested media types which select for Salmonella typhi, S. paratyphi A and B, S. schottmuelleri, Pseudomonas aeruginosa, beta and gamma hemolytic Streptococci, coagulase positive Staphylococcus aureus, Proteus species, Aspergillus species and other fungi, Candida albicans, coliforms, and Herellea species.

e) We made MLM studies with growth and detection cassettes containing filtration beds and:

1) mixed cultures, and

2) clinical specimens from human throat, skin, feces and urine.

Results demonstrated workability of the multiple filtration bed approach to determine organisms' probable numbers (relative numbers).

Our filtration bed approach also clarifies liquids and allows MLM organism detection in semi-solids, liquids, or wound, throat, and skin swabs.

f) Our freeze-dried culture media approach for preprogramming the growth and detection cassettes prove feasible and practical. Shelf life studies indicate that freeze-dried culture media can be stored for at least 4 months prior to use.

g) Our MLM electronic developments produced a working model which is called the Sequential Sampling Monitor, or SSAMM. Our working model proves capable of sequential viewing of an electro-optical detectors. Our arrangement permits future MLM system expansion. In addition, MLM operation has been substantially simplified by SSAMM.

h) Our tests also demonstrated that the entire filtration cassette can be frozen and preserved. All organisms tested were alive after 30 days at -79°C whereas 78 percent were alive after 60 days. The capability to preserve the cassettes and their living inhabitants expands MLM usefulness.

Our total progress with the MLM system has been outstanding and these new microbial detection concepts have great medical and industrial applications' potential.

2.0 INTRODUCTION

Difficulties with microbial contamination and minor infections of Apollo astronauts during flight have underscored the need for microbiological monitoring aboard manned spacecraft. The problem becomes more acute with longer missions. Even if problems had not occurred, the anticipated ecological changes during prolonged spaceflight should be established and monitored as a potential hazard.

Our McDonnell Douglas Astronautics Company-East (MDAC-East) developed Microbial Load Monitor (MLM) is designed to follow the ecological changes in spacecraft and astronaut microflora. It will aid in the establishment of baselines prior to developing microflora difficulties.

Our basic MLM concept involves culturing microorganisms in microchambers, then detecting microscopically small colonies with solid state electrooptics. By providing many channels of preprogrammed selective culture media, multiple data points can be collected for computer analysis. The result is rapid growth detection and an organism type prediction based on channels demonstrating positive growth or specific metabolic activity.

We evolved our original concept of growth channels imprinted in plastic tape into a rigid plastic card concept, similar to a computer punch card, containing windows for electrooptical detection. Each card is preprogrammed to accept certain specimen sample types and will be labeled "urine sample," "fecal sample," etc. The descriptive term card is used for simplicity. The card is actually a cassette containing dried media, filtration beds, and detection cells.

The test sample is inserted into a small automatic device having a slot for the plastic card of choice. After insertion, fluid transfer of the inoculum automatically fills media-containing card receptacles with sample fluid. Serial sample dilutions, clarification, and other necessary processes are automatically accomplished. The card is then inserted in the MLM, and colony growth detection occurs in a few hours. As stated, computer analysis predicts organisms kinds present. After growth and analysis, the card can be frozen and stored for future laboratory analysis.

Our total concept outlined above has been rapidly developed. We tested the selective culture media with several hundred actual hospital specimens. The totally integrated system, complete with automatic sampling devices, was

completed in a time permitting testing with human urine, fecal, skin, and throat clinical samples. Details and documentation of our developments form the bulk of this report.

3.0 METHODS AND RESULTS

3.1 Background

Our previous study contract, NAS 9-8329, demonstrated the feasibility of using combined electro-optical and selective culture techniques for rapidly detecting microbial groups and/or species. Mixed culture studies documented in the 30 June 1969 final report, established the following findings.

The most successful MLM tested wavelength was 900 nm. This wavelength was also shown to be optimum for the detection of organisms cellular masses; but was insensitivie to culture media color changes, and did not optimally detect precipitates occurring during growth. Spectrophotometric growth studies with a microcuvette indicated 665 nm superiority to 900 nm for precipitate detection and a mediums' color changes.

Several hundred culture media types and variations were screened for their selective ability on microorganisms groups or species in mixed populations. Selection was on a growth basis or growth plus a physiological event such as producing urease, coagulase, or pH change. In brief, the most successful selections from a mixed population were the following:

- Staphylococcus aureus with mannitol salt-DMSO-congo red medium
- Gamma Streptococcus with Mitis salivarius-sodium azide-congo red medium.
- Proteus species with urea magnesium phosphate medium.
- Pseudomonas aeruginosa with cadmium sulfate-nutrient broth medium.
- Corynebacterium diphtheria with potassium tellurite medium plus one percent bile.

Although the MLM demonstrated a capability of detecting microorganism growth in culture media, we also demonstrated that adding chemical gain indicators which precipitated with slight pH changes gave as much as seven-fold increased detection speed. Of those chemicals tested, we chose congo red and magnesium phosphate as most compatible with microorganisms. Congo red at 0.02 percent concentrations produces a blue-black precipitate on the acid side, (pH 6.5 - 6.0), while 1.0 percent magnesium sulfate produces a white milky precipitate on the basic side, (pH 7.4 - 8.0).

Aided by the above precipitation type indicators, carbohydrate utilization was rapidly detected by the MLM. Test tube studies with several Proteus species indicated that urease production should be rapidly MLM detected, but we did not perform such instrument tests.

Limited success or insignificant accomplishment areas were the fungi growth and separation, tape culture anaerobe growth and no technique development to detect coagulase production.

However, we satisfied that contract objective by demonstrating MLM concept feasibility.

3.2 Object of the Current Contract

Our current contract objective is to develop the MLM concept and hardware to the stage that the MLM can function as a diagnostic instrument capable of detecting medically important organisms in clinical specimens.

We contracted to perform the following tasks taken directly from the contract Statement of Work.

3.2.1 Technology Development -

a) Evaluate previously developed procedures providing data necessary for specific microorganism identification in mixed cultures. These studies will determine the reliability of these procedures for differentiation when the variables are physical growth conditions, inoculum size, and culture media.

b) Develop MLM isolation and identification techniques for selected organisms. Refine those techniques previously developed. Specifically study microorganisms identified as medically important, which include, but are not limited to the following:

- Streptococcus - beta hemolytic
- Staphylococcus (coagulase positive)
- Diplococcus pneumoniae
- Pseudomonas aeruginosa
- Salmonella typhosa
- Proteus morganii

- Neisseria meningitidis
- Mima polymorpha
- Herellea species
- Escherichia coli
- Klebsiella - Aerobacter
- Candida albicans
- Aspergillus species

c) Evaluate, and modify as required, all procedures using mixed cultures.

d) Develop methods and procedures for determining counts, or relative test microorganism numbers. These studies include, but are not limited to, an evaluation of techniques such as growth rate and turbidity coefficient concepts previously reports.

3.2.2 Detector Development - Develop detector sampling devices and make minor modifications to improve MLM performance.

a) Design, fabricate, and evaluate performance of a functional model, or models, to provide microbiological sampling of liquids and semi-solids for the MLM.

b) Develop and test rigid multichannel sample holders.

c) Design and test minor MLM modifications which are determined necessary to improve microorganism identification capabilities.

3.2.3 Evaluate MLM Performance and Perform Experimental Studies -

a) Evaluate identification procedures using throat, urine, and fecal samples seeded with test organisms to establish those techniques which can be used in an MLM flight version. We will also develop logic data necessary for identification of the test organisms.

b) Evaluate identification procedures using naturally occurring organisms to establish false positive probabilities with selected isolation techniques.

c) Test and evaluate the performance of MLM sampling devices.

3.3 Results of Technology Development Studies

We divided the biological technology development into four major efforts. These were (a) evaluating previously developed procedures to provide necessary mixed culture study data; (b) developing techniques for isolating and identifying organisms NASA chosen as medically important, and for which no techniques were available; (c) evaluate and modify as required all procedures involving mixed cultures; and (d) developing methods and procedures for determining test organism counts or relative numbers. The following discussion expounds our study data and results.

3.3.1 Evaluation of Previously Developed Techniques - We evaluated the engineering and microbiological developments under contract NAS 9-8329, Manned Spacecraft Microbial Load Monitor, in the contractual first quarter. Improvement areas and further study were identified. These data served as new approach baseline information as well as refinements on mixed culture study.

3.3.2 Laboratory MLM Instrument - We accomplished laboratory testing with a six channel detector of electronic design similar to the NASA MSC engineering model delivered in September 1969. Six channels operated at 665 nm while the remaining two operated at 900 nm. We designed the detectors to accommodate rigid plastic growth chambers similar to the type we recommended in the contract NAS 9-8329 final report. Figure 1 photographically depicts the detectors. The photograph displays panel slots where extra channels may be placed between the viewed detectors. This represents a versatility which allows interchange of various wavelength detectors. The rigid plastic growth cells used were type similar as presented in Figure 2 and were equipped with optional fliter beds, descriptively reported later.

3.3.3 Culture Media Precipitation Indicators- In previous work we established that precipitation type indicators gave a significant sensitivity increase and resultant decrease in growth detection time required. Congo red was given as the choice precipitation type indicator signifying pH 6.5 or less. However, we performed no large scale tests to determine congo red toxicity for medically important microorganisms.

Tests we performed in the current contract first three weeks uncovered evidence of congo red inhibiting certain organisms, namely: Herellea species,

FIGURE 1
LABORATORY VERSION OF THE MICROBIAL LOAD MONITOR CURRENTLY IN USE IN THE MICROBIOLOGY LABORATORY

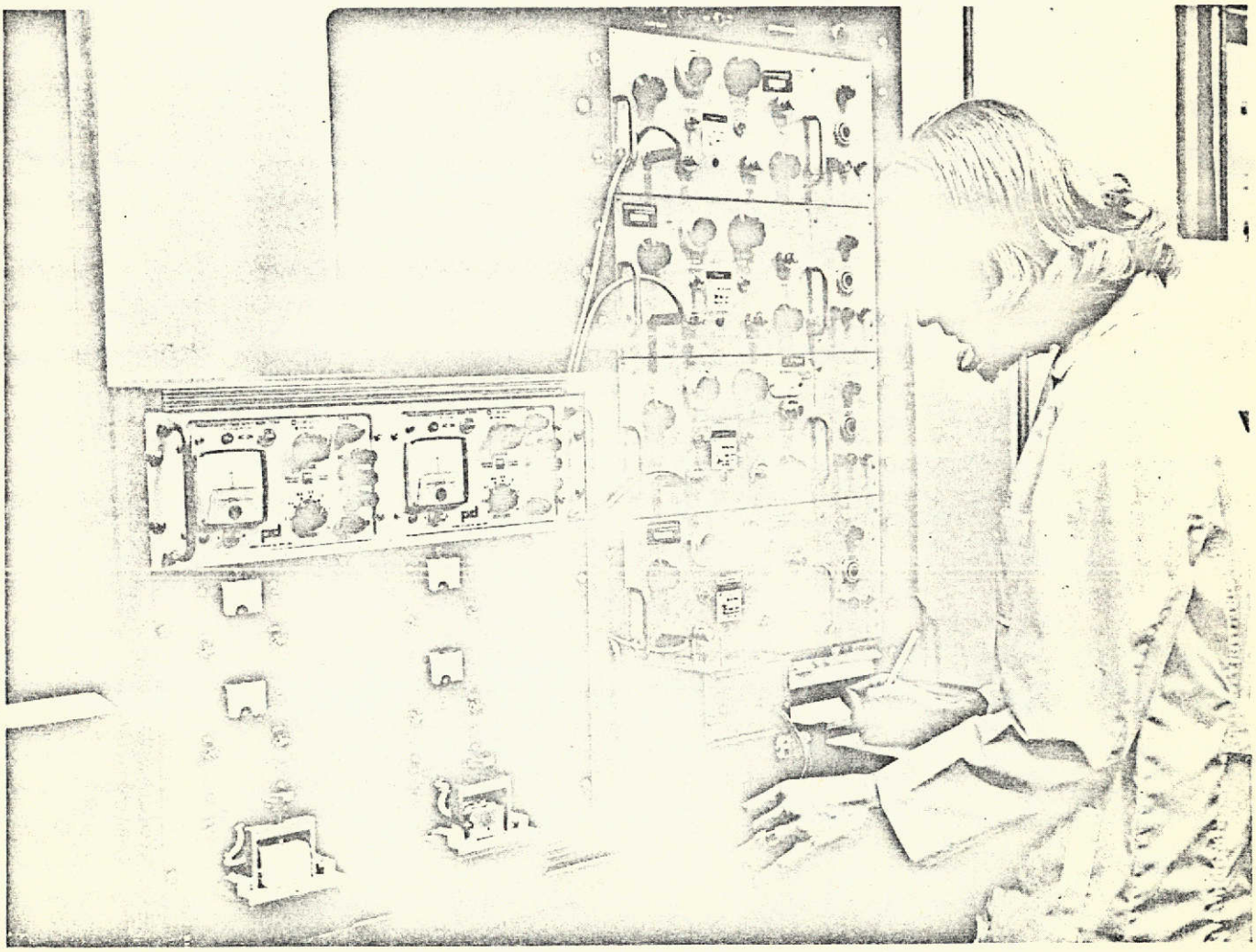
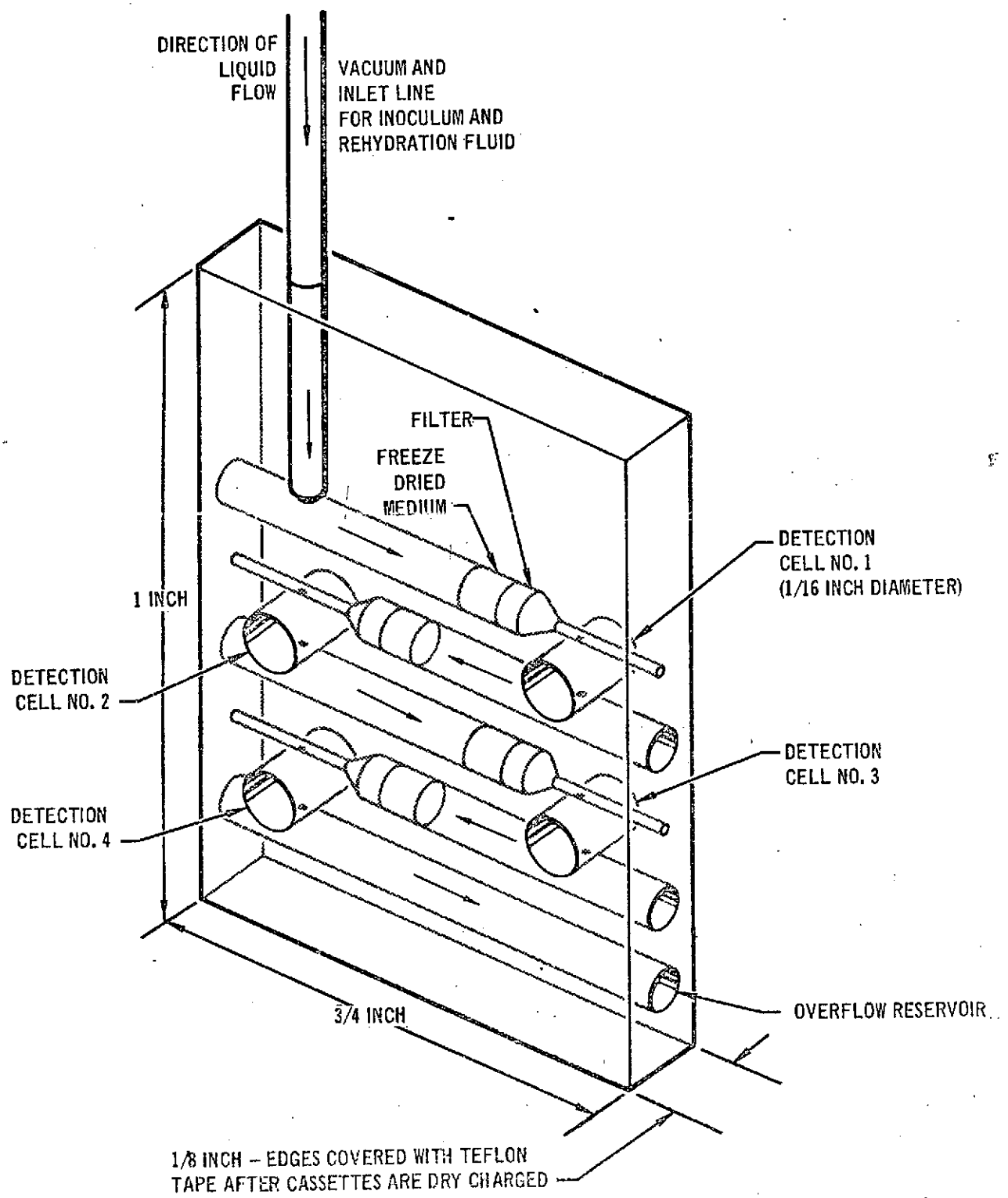


FIGURE 2
FILTER CASSETTE PRE-PROGRAMMED WITH FREEZE DRIED MEDIUM



Proteus species, and Streptococcus species. All three organisms are found on the NASA supplied medically important organisms' list: We therefore abandoned congo red due to the described difficulties.

3.3.4 Evaluating Previously Developed Mixed Culture Selective Media - We reported literature describing absolute selection of Pseudomonas aeruginosa from all other organisms. This method, described in the previous contract final report, used cadmium sulfate. Work in our laboratory confirmed the claim, however, growth of Pseudomonas aeruginosa is so retarded that 40 hours are required for growth detection. Therefore, we evaluated another culture medium: cetrimide broth, with the MLM.

The chemical name for cetrimide is cetyl trimethyl ammonium bromide.[2] Cetrimide has been used for selection of Pseudomonas aeruginosa in various culture media. The MLM formula selected is as follows:

Tryptic soy broth	30.0 g/l
Cetrimide	0.2 g/l

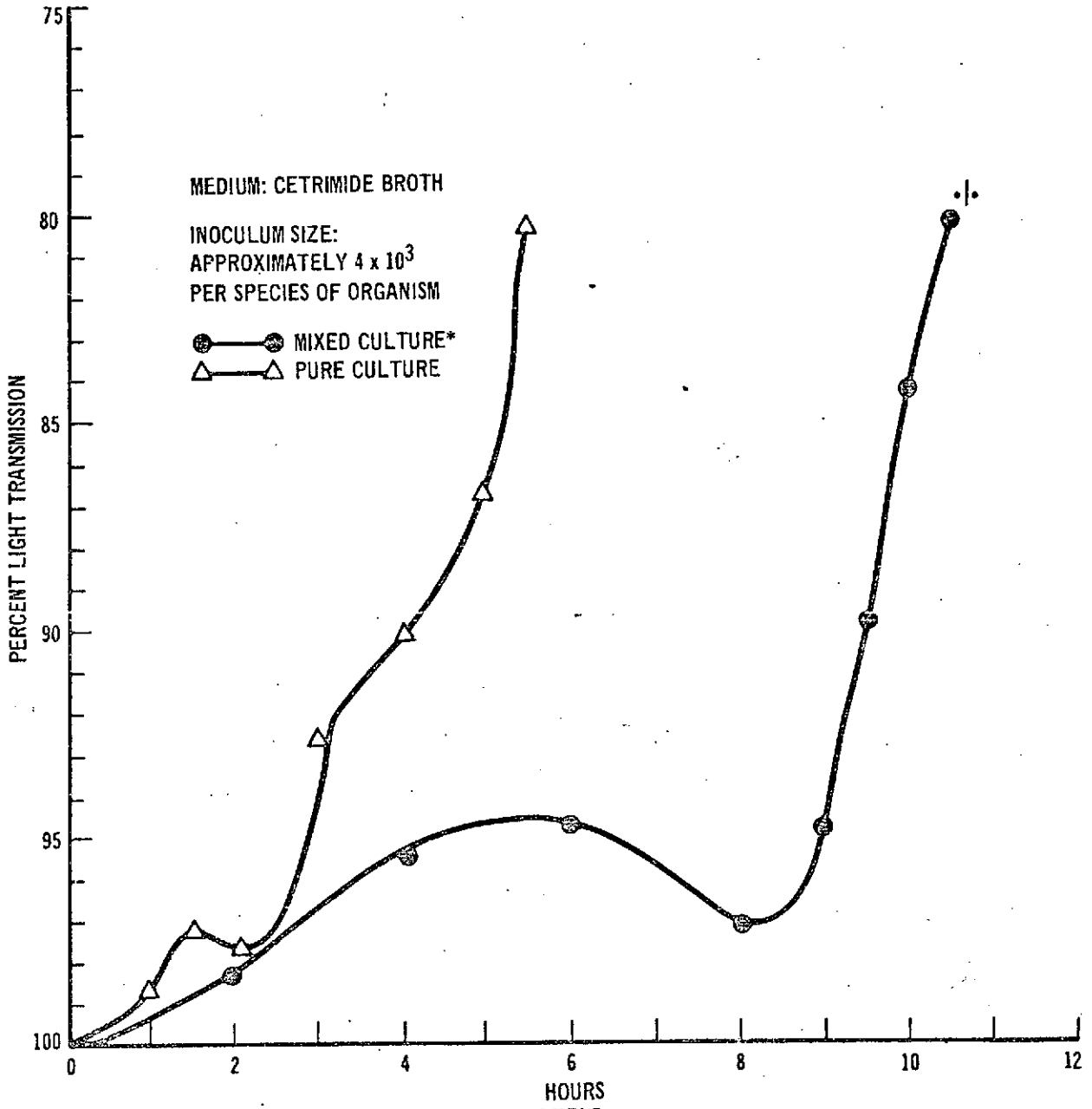
Ingredients are water dissolved and heated to boiling. The final pH is 7.2 and filtration sterilization is accomplished. Selection with this formula is absolute. No other organisms tested and no other Pseudomonas grow except aeruginosa species. Fortunately the growth of Pseudomonas aeruginosa is not greatly inhibited.

Figure 3 presents MLM test results with this culture medium at 665 nm. Note the curious growth curve hump which occurs. It is consistent and corresponds to the blue-green pigment appearance. Our first test results indicated that Pseudomonas aeruginosa could be MLM detected from mixtures containing at least 22 different microorganism kinds.

Our earlier test tube trials indicated that urease activity could be MLM detected, but no such MLM tests had actually been performed.

First quarter testing revealed that the original formula recommended in the previous contract final report was inadequate in two areas: Precipitation was found to occur only when the inoculum size was large, and some strains of Proteus vulgaris were found which would not grow in the medium in less than 48 hours. We tested thirty different magnesium sulfate indicator modifications with the medium. Such modification examples are the substitution of tannic

FIGURE 3
MLM DETECTION OF PSEUDOMONAS AERUGINOSA IN PURE AND MIXED CULTURES.
WAVELENGTH WAS 665 NANOMETERS



·|· ONLY PSEUDOMONAS ISOLATED AT COMPLETION OF TEST
*ORGANISMS IN MIXTURE

1. ESCHERICHIA COLI
2. AEROBACTER AEROGENES
3. KLEBSIELLA PNEUMONIAE
4. PROTEUS MORGANII
5. PSEUDOMONAS AERUGINOSA
6. SERRATIA MARCESCENS
7. HERELLEA SPECIES
8. SHIGELLA FLEXNERI
9. SALMONELLA TYPHOSA
10. NOCARDIA CORALLINA
11. STAPHYLOCOCCUS AUREUS
12. MICROCOCCUS RUBENS
13. SARCINA LUTEA
14. γ STREPTOCOCCUS
15. BACILLUS SUBTILIS
16. CANDIDA ALBICANS
17. ASPERGILLUS NIGER
18. SACCHAROMYCES CEREVISIAE
19. ALCALIGENES FAECALIS
20. ENTEROBACTER SPECIES
21. CITROBACTER SPECIES
22. PROVIDENCE SPECIES

acid complexed with calcium pantothenate; leucine addition to the urease medium; and buffer concentration changes. The evolved formula is as follows:

Urease Detection Medium

Glucose	1.0 g/l
Gelysate	2.0 g/l
Urea	30.0 g/l
KH_2PO_4	1.4 g/l
K_2HPO_4	1.0 g/l
NaCl	5.0 g/l
$MgSO_4$	10.0 g/l

Final pH is 6.3. The medium is filtration sterilized.

We proved this formula successful for all Proteus strains tested which include 3 recent Proteus isolates from local hospitals. In this medium magnesium phosphate is formed from magnesium sulfate and phosphate buffer. Precipitation occurs at pH 7.2 - 7.4 and completed at pH 8.0. However, as the pH rises, the crystals' characteristics change. These changes are MLM recorded.

When we buffered the urease medium with only K_2HPO_4 the pH rise was quite rapid, as would be expected. However, with increasing pH the crystals change in appearance, yielding a sudden light transmission increase. The standard urease test medium routinely used in clinical laboratory work is buffered with KH_2PO_4 to assist differentiating those organisms which are weakly urease positive, such as Klebsiella and the strongly positive Proteae. The Klebsiella organisms cannot overcome the buffer and the reaction remains negative. However, because the crystals become less opaque with increasing pH it is possible to differentiate the Proteae and weakly reactive Klebsiella Aerobacter organisms. Figure 4 illustrates this fact. As the graph shows, the precipitation seems to dissolve, resulting in a light transmission increase. However, microscopic examination reveals that the crystals have changed from amorphous to definite flat polygons. By altering buffer concentration, it is possible to differentiate Proteus species. This is because there is a difference in urease activity with resultant limited total alkalinity produced. Figure 5 illustrates crystal production differences in the same medium by different Proteus species.

FIGURE 4
MLM DETECTION OF UREASE ACTIVITY IN PROTEUS MIRABILIS
WAVELENGTH WAS 665 NANOMETERS

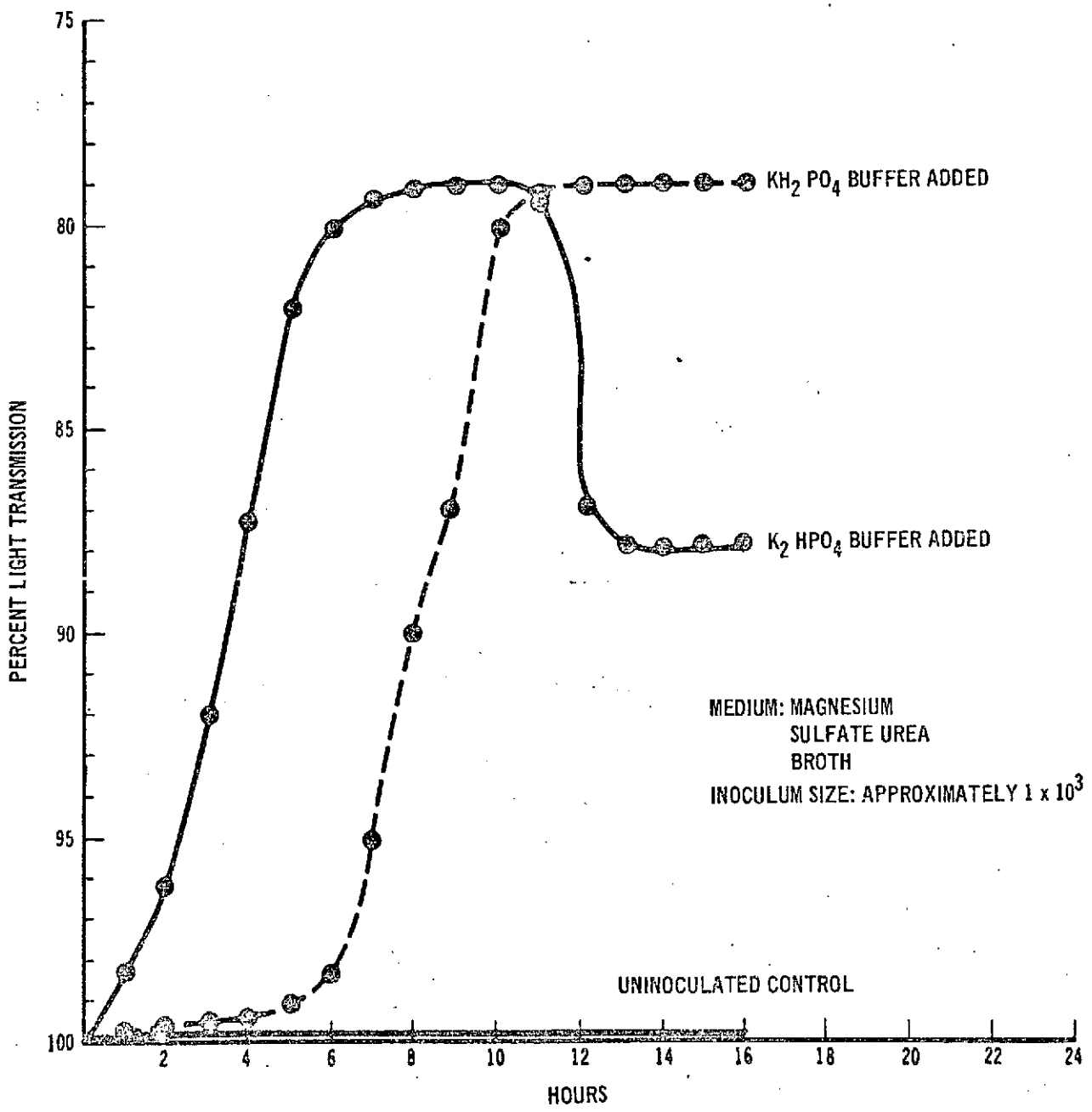
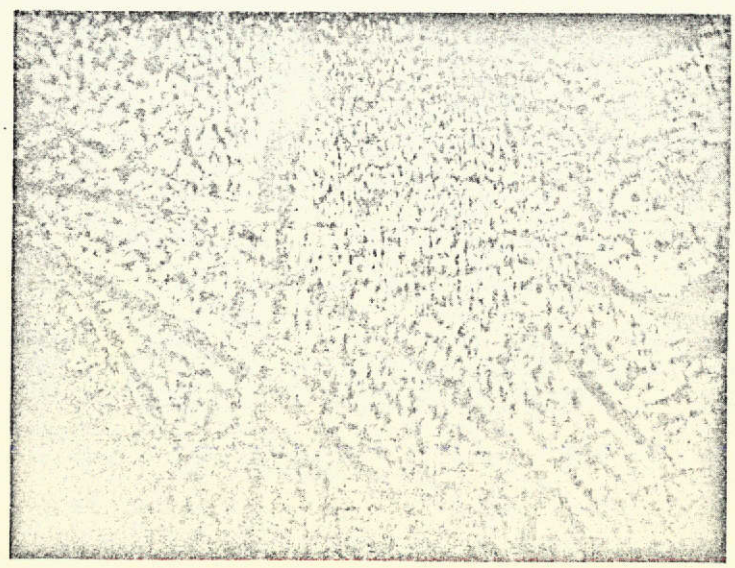


FIGURE 5
EFFECT OF SPECIES VARIATION ON TYPE OF PRECIPITATE FORMED IN UREASE CULTURE MEDIUM

A
PHOTOMICROGRAPH OF PRECIPITATE FORMED BY PROTEUS VULGARIS (X 100)

B
PHOTOMICROGRAPH OF PRECIPITATE FORMED BY PROTEUS MORGANII IN SAME MEDIUM AS ABOVE (S 100)



Thus it is possible for urease production with rapid MLM detection. We detected Proteus species by the curve changes which can only occur in the presence of strongly reactive Proteus species. In actual MLM tests, the Klebsiella - Aerobacter group yields a curve identifcal to the one labeled "K_2HPO_4 added" except for the dip characteristic of all Proteae tested.

Our previous contract final report recommended Mannitol-Salt-DMSO culture medium. Further evaluation revealed a disadvantage of being too toxic even for staphylococci. Maintaining 17 percent DMSO is very difficult due to its hygroscopic nature. One percent variation can be too toxic for some strains. MLM detection time is greater than with most other selective media. Furthermore there is no way to differentiate coagulase positive and coagulase negative staphylococci in this medium. Tests with coagulase negative staphylococci skin isolates yielded better and faster growth than any other strains. The medium contained congo red as a precipitation type indicator and detection was based upon mannitol utilization producing acid.

We decided to try a different approach while incorporating some of the recommended medium advantages.

We performed MLM tests to determine if producing a coagulum could be detected as a precipitation type indicator. This feasibility was demonstrated but problems with blood plasma instantly developed. Reproducibility was not possible. The medium was super sensitive to aging, pH change, and inoculum size.

It was therefore decided to switch from plasma to egg yolk as a coagulum protein source. It has been reported that approximately 90 percent of all coagulase positive staphylococci also precipitate egg yolk.[3] After some modifications, we developed the following medium for MLM use.

Staphylococcus Coagulase Detection Medium

Beef extract	1.0 g/l
Polypeptone	10.0 g/l
NaCl	75.0 g/l
D-Mannitol	10.0 g/l
Egg Yolk	70.0 g/l
Potassium Tellurite, 1.0 ml of 1% solution	

The medium is filter sterilized.

Tests with 20 different genera revealed that all staphylococci tested rapidly grew. Coagulase positive organisms produced coagulum in less than four hours, (inoculum size dependent.) However, gamma Streptococcus also grew in the medium but produced no coagulum or precipitate. All other genera failed to grow in less than 18 hours, (inoculum size independent.)

We used a mixture of Staphylococcus aureus and gamma Streptococcus to test this medium. Figure 6 presents the results. In spite of Streptococcus growth, the MLM rapidly detected only the caogulase positive Staphylococcus which form a precipitate in this medium. We also recorded coagulase negative Staphylococcus and gamma Streptococcus growth, but these organisms require approximately twice the Staphylococcus growth time depending upon inoculum size. Figure 6 notes coagulase production resulting in full scale readings in approximately 4 hours. Inoculum size was approximately 1×10^4 viable organisms for each species.

Evaluating previously tested selenite congo red broth reveals the medium as described in the previous contract final report required modification. To be effective, selenite broth must remain near neutrality. Congo red addition gains little because congo red does not precipitate until the acid reaction reaches pH 6.5 or less. We originally supposed that selenite would select for Salmonella or Shigella species and then their carbohydrate utilization could be detected by congo red precipitation. However, selenite precipitation also occurs at pH values higher than 7.0, therefore the medium reaction cannot be MLM determined with this combination.

We developed and MLM tested a new coliform medium. The basic medium is standard Brilliant Green Bile medium in which 1.0 percent sodium desoxycholate replaces the oxgall. This percentage of sodium desoxycholate is acid sensitive and will begin precipitation at pH 6.5 and completed at pH 5.0. This medium is quite selective for lactose fermenting organisms. Of 34 different species tested only Escherichia coli, Aerobacter aerogenes, Klebsiella pneumoniae, Citrobacter species and Enterobacter liquefaciens were found to grow with desoxycholate resultant precipitation. In Figure 7 the graph notes coliform detection is quite rapid while other organism mixtures remain undetected. It is interesting to note that the oxgall normally used in Brilliant Green Bile medium will not precipitate with pH changes.

FIGURE 6
MLM DETECTION OF COAGULASE POSITIVE STAPHYLOCOCCUS AND DIFFERENTATION FROM GAMMA STREPTOCOCCUS WHICH GROWS IN CULTURE MEDIUM
WAVELENGTH WAS 665 NANOMETERS

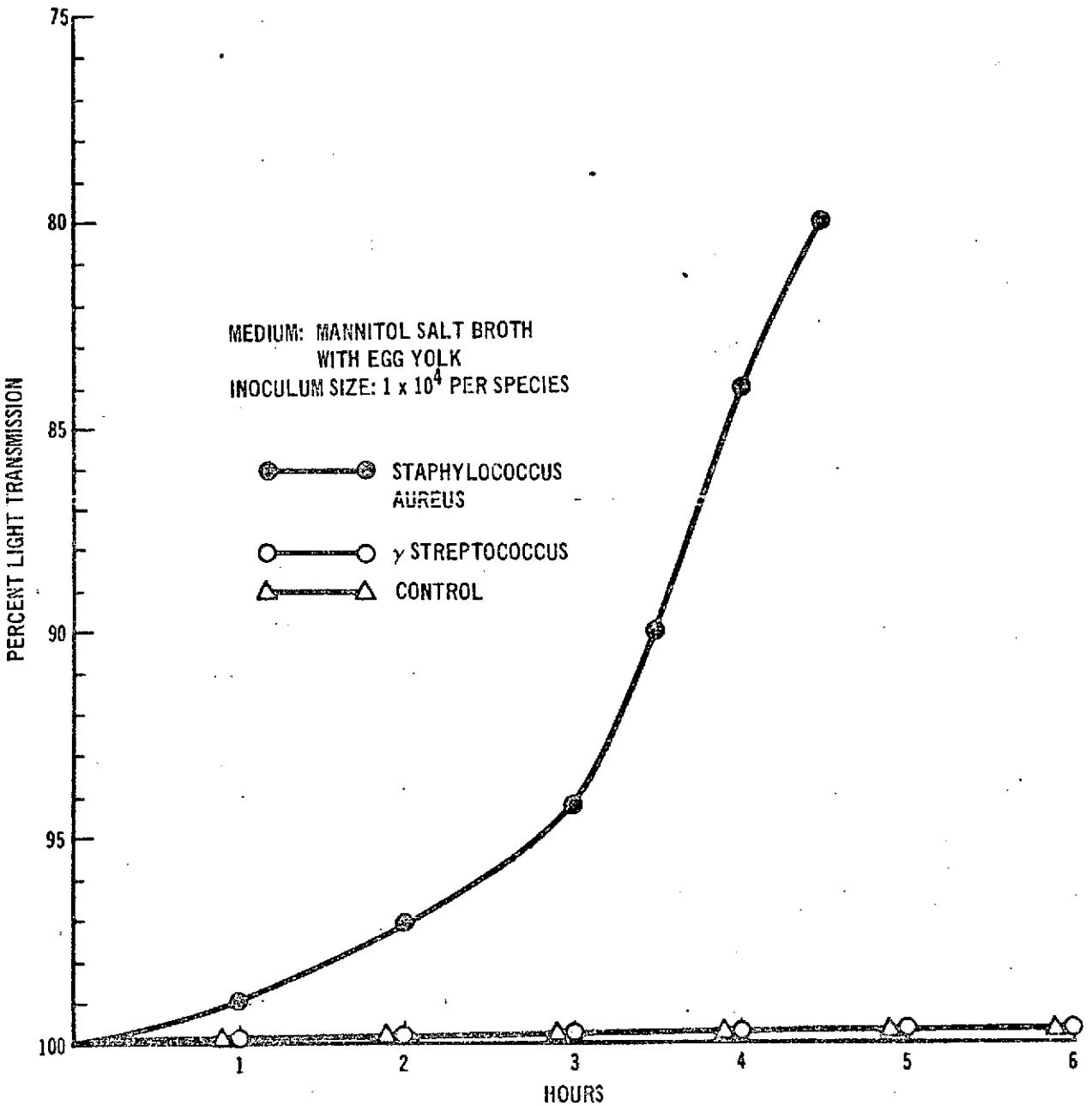
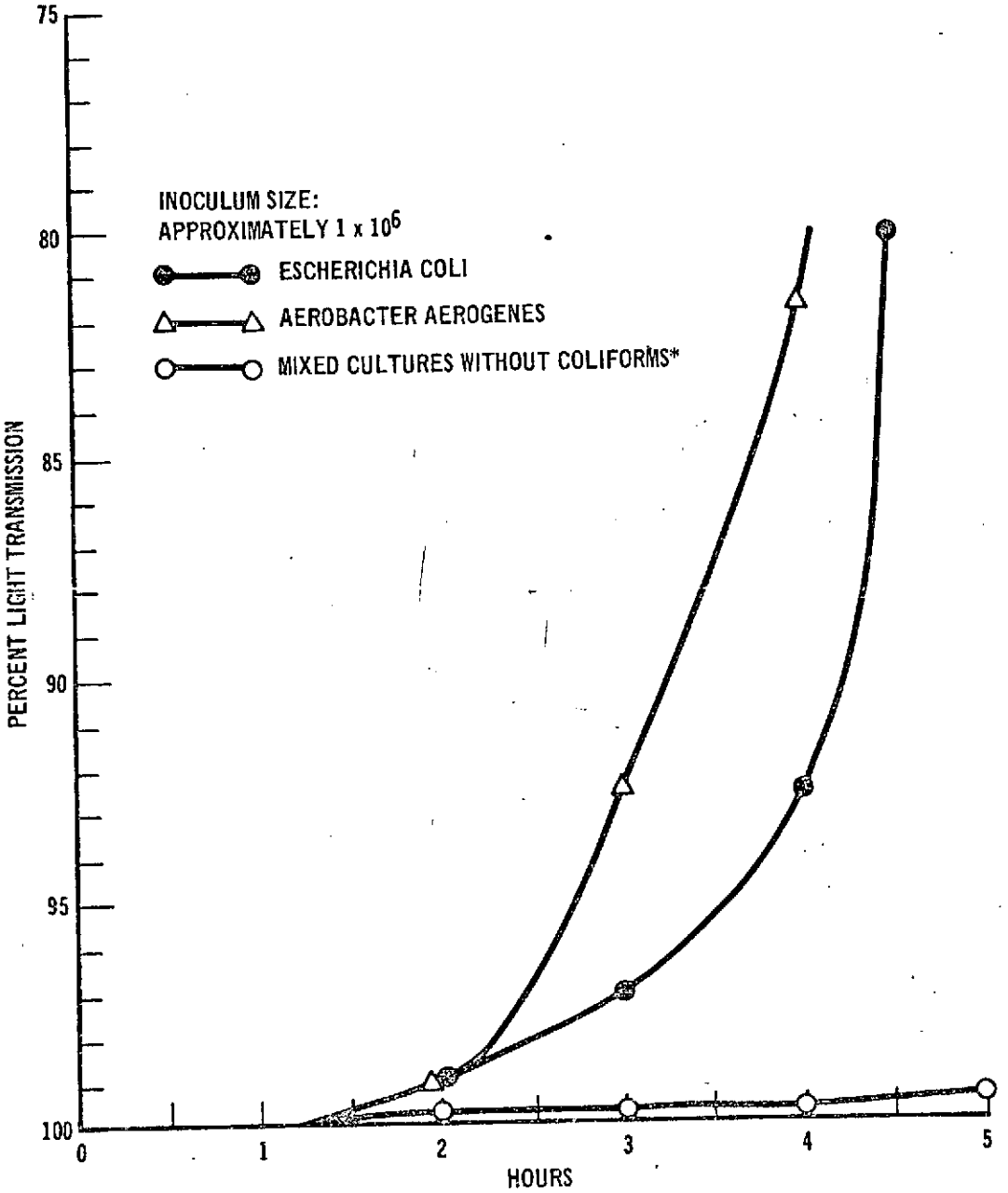


FIGURE 7
MLM DETECTION OF COLIFORMS AND DISCRIMINATION AGAINST EIGHTEEN OTHER ORGANISMS IN COLIFORM BROTH



* MIXTURE CONTAINED:

STAPHYLOCOCCUS AUREUS
HERELLEA SPECIES
ALCALIGENES FAECALIS
PROVIDENCE SPECIES
STAPHYLOCOCCUS EPIDERMIDIS
MICROCOCCUS RUBENS
SERRATIA MARCESCENS
NOCARDIA CORALLINA
SACCHAROMYCES CEREVISIAE
PROTEUS RETTGERI
SALMONELLA TYPHOSA
SARCINA LUTEA
PSEUDOMONUS AERUGINOSA
SHIGELLA SONNEI
GAFFKYA TETRAGENA
GAMMA STREPTOCOCCUS

3.3.5 Isolation and Identification Technique Developments for Selected Organisms and for Which no Technique is Currently Available - Although our previous work was unsuccessful, first quarter testing yielded two media for the successful MLM fungi detection. We successfully grew Candida albicans and Aspergillus niger in microculture and detection occurred in a few hours.

At first it was thought that a chemical gain or precipitation type indicator would be necessary for the early fungi detection. However, in light of congo red possible toxicity, it was decided to MLM test the medium without any indicator. Fungi are much larger than bacteria and we hoped that we would detect cell mass alone. The Candida albicans medium was Mycosel. The Mycosel broth formula is as follows:

Mycosel Broth

Phytone peptone	10.0 g/l
Dextrose	10.0 g/l
Cycloheximide	0.4 g/l
Chloramphenicol	.05 g/l
Final pH is 6.9	

Ingredients were dissolved without heating and filter sterilized.

The Aspergillus niger medium was a synthetic broth which is a standard Czapek Dox Broth modification. Our modification is omitting ferrous sulfate and adding phenol red indicator. The formula is as follows:

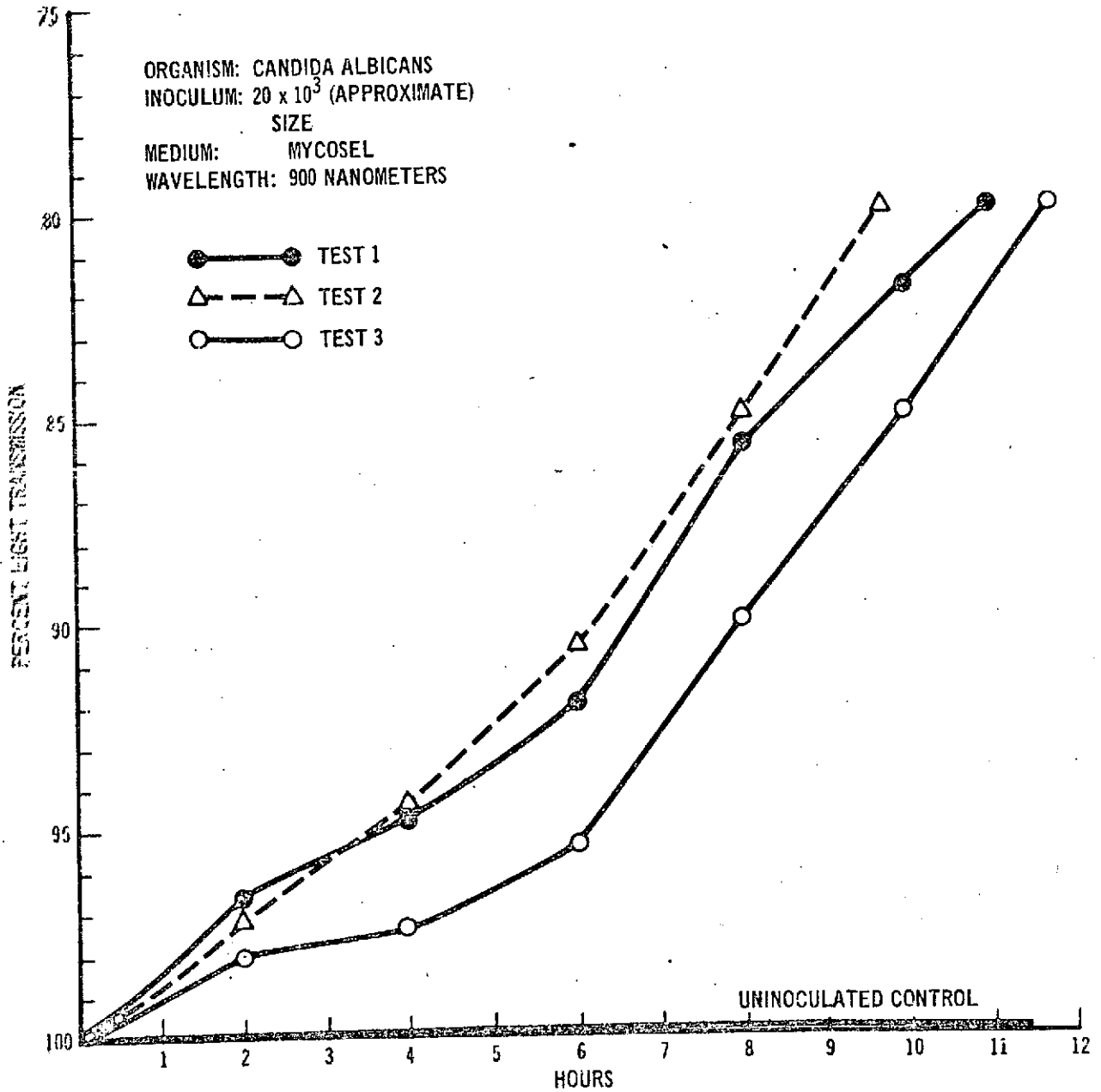
Aspergillus Niger Medium

Sucrose	30.0 g/l
$NaNO_3$	3.0 g/l
K_2HPO_4	1.0 g/l
$MgSO_4$	0.5 g/l
KCl	0.5 g/l
Phenol Red	0.025 g/l
Final pH 7.3	

Ingredients were dissolved without heating, and filter sterilized.

Figure 8 presents Candida albicans detection results at 900 nm. We determined inoculum size (approximately 2.0×10^3) by pour plate assay in Sabouraud's dextrose agar. Full scale deflection (20 percent change in light transmission)

FIGURE 8
MLM DETECTION OF GROWTH OF FUNGI WITHOUT CHEMICAL GAIN INDICATORS



occurred in three trials in 12 hours or less. Aspergillus niger results were also better than expected. We used two wavelengths, 665 and 900 nm, for these tests. The fungi culture medium was a synthetic broth. Figure 9 presents the results. We observe a gross time difference required to achieve a 20 percent light transmission drop. At 665 nm, the time is between 10 and 11 hours. At 900 nm, 54 hours are required to reach the same light transmission. Inoculum size and all conditions save wavelengths were identical. Incubation temperature was 35°C in all MLM tests performed.

It is apparent that 665 nanometers is the superior wavelength for detecting Aspergillus niger. We questioned, is 665 nm superior for detecting all fungi?

We performed further tests with Candida albicans. In this test series, inoculum size and wavelength were the variables. Figure 10 presents the results. We reduced inoculum size as low as is practical. When inoculum size was less than six thousand organisms per culture chamber, growth reduced light transmission to the 20 percent mark in less than 10 hours at 665 nm versus 12 hours at 900 nm. When inoculum size was approximately 20 viable cells per growth chamber (detection cell) the 20 percent light reduction mark was achieved in about 15 hours at 665 nm versus 18 hours at 900 nm. These results reveal a clear 665 nm advantage for the fungi detection without a precipitation type indicator.

Fungi growth documentation in the detection cell growth chambers is provided by Candida albicans and Aspergillus niger photomicrographs. Results before and after growth are presented in Figure 11 and 12. Cell morphology is not grossly different from that seen in usual broth culture with both organisms.

Mixed culture studies with 22 different organisms added to Mycosel broth revealed that only Candida albicans and some pathogenic dermatophytes grew in 24 hours or less. Other fungi, including yeasts, did not grow. We tested synthetic Aspergillus niger broth against 22 microorganisms. Of those tested only Aspergillus niger and Candida albicans were found to grow in this medium and Candida did not grow as rapidly as Aspergillus. We performed additional screening tests with 28 known fungi, most of which were obtained from American Type Culture Collection (ATCC). Table 1 presents the results.

FIGURE 9
MLM DETECTION OF GROWTH OF FUNGI WITHOUT CHEMICAL GAIN INDICATORS – EFFECT OF WAVELENGTH ON DETECTION OF ASPERGILLUS NIGER

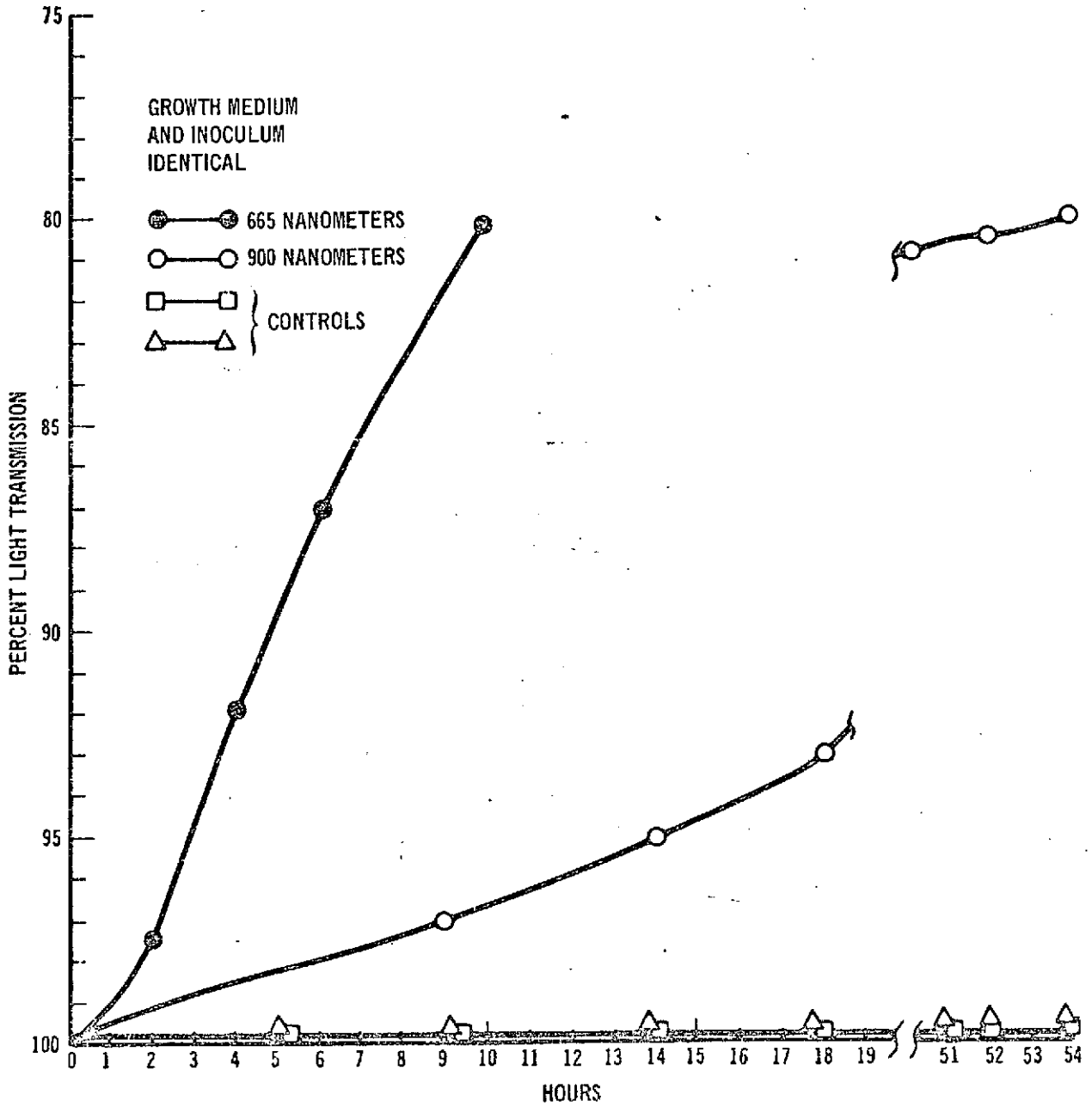


FIGURE 10
MLM DETECTION OF GROWTH OF FUNGI WITHOUT CHEMICAL GAIN INDICATORS – COMPARISON OF WAVELENGTH EFFECT AND INOCULUM SIZE ON CANDIDA ALBICANS

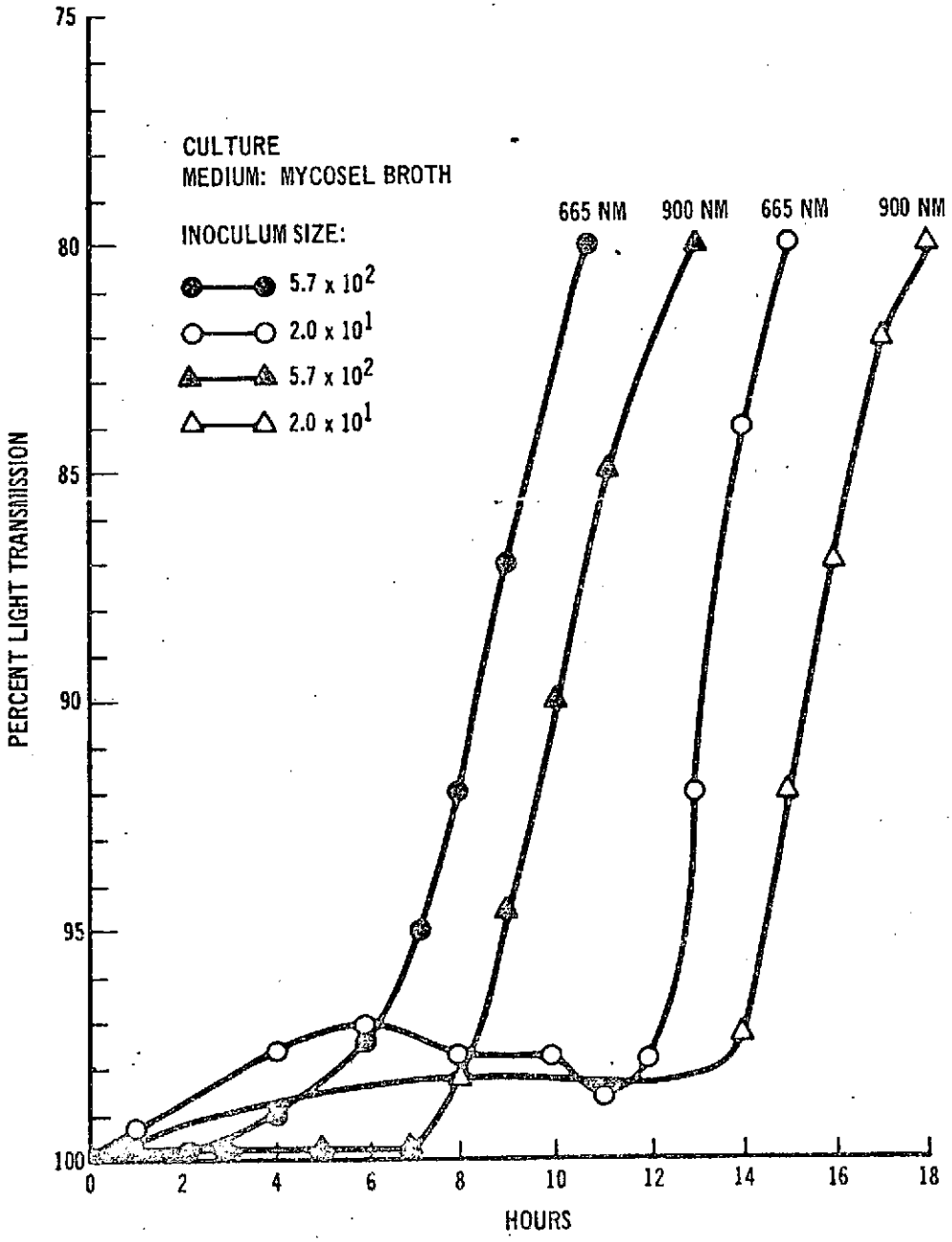
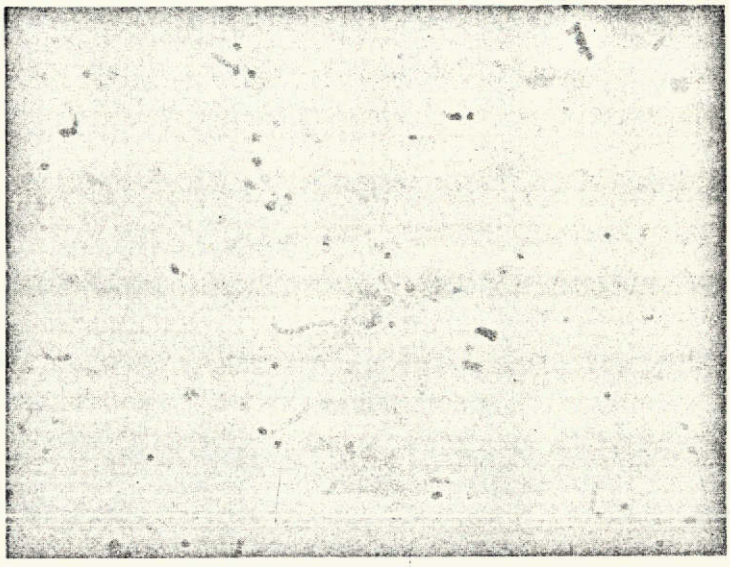


FIGURE 11
GROWTH OF ASPERGILLUS NIGER IN MLM GROWTH CHAMBERS.
CULTURE MEDIUM WAS SYNTHETIC FOR FUNGI

A

PHOTOMICROGRAPH OF GROWTH CHAMBER IMMEDIATELY
FOLLOWING INOCULATION (X 400)



B

PHOTOMICROGRAPH CHAMBER AFTER FULL SCALE DETECTION
OF GROWTH IN MLM (X 400)

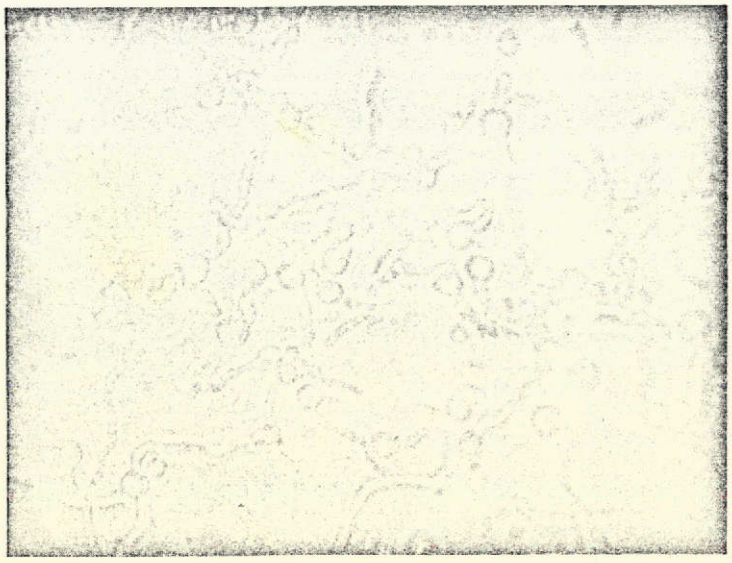
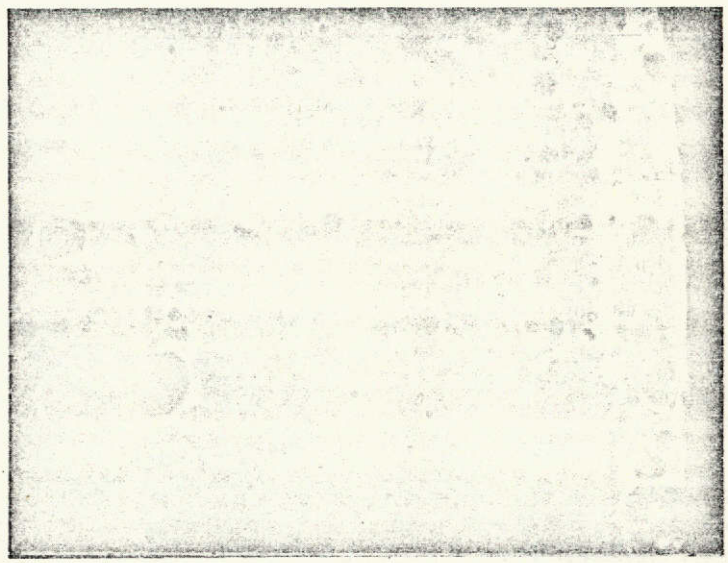


FIGURE 12
GROWTH OF CANDIDA ALBICANS IN MLM GROWTH CHAMBER
CULTURE MEDIUM WAS MYCOSEL

A
PHOTOMICROGRAPH OF GROWTH CHAMBER
IMMEDIATELY FOLLOWING INSTRUCTIONS (X 400)



B
PHOTOMICROGRAPH OF ABOVE AFTER FULL
SCALE DETECTION OF GROWTH IN MLM (X 400)

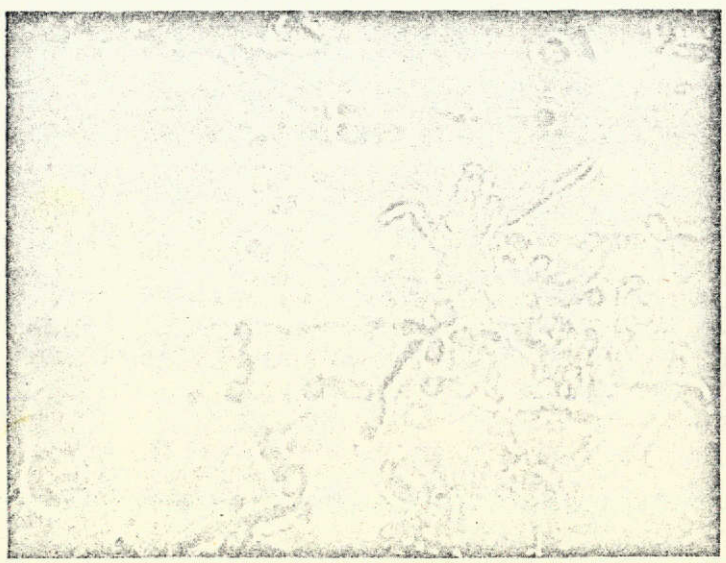


TABLE 1
SELECTIVITY TESTS FOR MLM MEDIA FOR FUNGI

EACH OF THE FOLLOWING ORGANISMS WAS INOCULATED INTO CANDIDA AND ASPERGILLUS MEDIA DEVELOPED FOR THE MLM. INCUBATION WAS AT 37°C, OBSERVATIONS WERE MADE AT 16 AND 48 HR.

ATCC NO.	ORGANISM	SYNTHETIC BROTH FUNGI 16 HR	SYNTHETIC BROTH FUNGI 48 HR	MYCOSEL BROTH 16 HR	MYCOSEL BROTH 48 HR
–*	ALTERNARIA SPECIES	0	0	0	0
–*	ASPERGILLUS NIGER	+	+	0	0
–	ASPERGILLUS NIGER	+	+	0	0
14110	ASPERGILLUS FUMIGATUS	0	±	0	0
16619	ASPERGILLUS FUMIGATUS	0	±	0	0
11516	ABSIDIA CYLINDROSPORA	0	0	0	0
14053	CANDIDA ALBICANS	0	0	+	+
11006	CANDIDA STELLATOIDEA	0	±	+	+
2512	CANDIDA PSEUDOTROPICALIS	0	±	+	+
750	CANDIDA TROPICALIS	0	0	0	0
9506	CRYPTOCOCCUS NEOFORMANA	0	0	0	0
659	FUSARIUM OXYSPORUM	0	0	0	0
755	GEOTRICHUM CANDIDUM	0	0	0	±
14556	MONILIA GEOPHILA	0	0	0	0
–*	MUCOR SPECIMEN	0	0	0	0
15087	MUCOR AZYGOSPORA	0	0	0	0
11621	MICROSPORUM CANIS	0	0	0	0
9083	MICROSPORUM GYPSEUM	0	0	0	0
9277	NEUROSPORA CRASSA	0	0	0	0
–*	PENICILLIUM SPECIES	±	+	0	0
16965	PENICILLIUM BREVI-COMPACTUN	0	±	0	0
8537	PENICILLIUM NOTATUM	+	+	0	0
–*	RHIZOPUS SPECIES	0	0	0	0
6204	RHIZOPUS ARRHIZUS	0	0	0	0
14084	SACCHAROMYCES ACETI	0	0	0	0
10212	SPOROTHRIX SHENCKII	0	0	0	±
4807	TRICHOPHYTON MENTAGRAPHYTES	0	0	0	0
10218	TRICHOPHYTON RUBRUM	0	0	0	0

+ = HEAVY GROWTH
± = VERY LIGHT TO LIGHT GROWTH
0 = NO GROWTH
* OBTAINED FROM STOCKS, UNIVERSITY OF OKLAHOMA

These tests established that fungi could be MLM detected, and that 665 nm was adequate to detect fungi without an indicator.

We screened several dozen media in our laboratory for possible MLM application to medically important bacteria.

We rapidly accomplished screening tests by utilizing sterile plastic plates containing many rows of small wells. Tissue culture microtest plates were most often used. We covered the wells with sterile transparent adhesive tape, and rapidly filled them with sterile culture broth by puncturing the tape with a syringe and needle. We performed serial dilutions similarly by aseptically puncturing successive wells for appropriate culture mixing and withdrawal. Such screening test results are presented in Table 2. The difference between the observed MLM detected changes are also presented. Usually, MLM detection precedes by at least a few hours the human eye observable changes. However, this is not the case with Aspergillus niger detection as is noted in the Table.

Candidate culture media are chosen by utilizing literature search, experience, consultation with colleagues, recommendations by our consultant, Dr. J. B. Clark, and some guesses based upon combining all the above.

During the evaluation of previously reported culture media, we made observations leading to developing improved selective media for the following organisms:

a) Coagulase positive Staphylococcus aureus.

b) Pseudomonas aeruginosa.

c) Proteus species.

d) Candida albicans

e) Aspergillus niger

f) Coliform organisms

Remaining organisms requiring selective media development for MLM detection are as follows:

TABLE 2

EFFECT OF INOCULUM SIZE ON LENGTH OF TIME REQUIRED TO PRODUCE OBSERVABLE CHANGES IN CULTURE MEDIUM

TEST WAS PERFORMED IN TISSUE CULTURE MICROPLATES AT 35°C. DETECTION OF GROWTH WAS BY HUMAN EYE. TOTAL VOLUME OF CULTURE MEDIUM WAS 0.3 ML.				TESTS PERFORMED IN MLM. TOTAL VOLUME 0.01 ML. WAVELENGTH 665 NM
MEDIUM	ORGANISM	INOCULUM SIZE (APPROXIMATE)	HOURS TO OBSERVABLE CHANGE	HOURS TO FULL SCALE DETECTION IN MLM
CETRIMIDE BROTH	PSEUDOMONAS AERUGINOSA (BROWN PIGMENT STRAIN)	1×10^7	7	
		1×10^6	12	
		1×10^5	12	
		1×10^4	13	
		1×10^3	16	
		1×10^2	16	
		1×10^1	17	
	PSEUDOMONAS AERUGINOSA (GREEN PIGMENT STRAIN)	1×10^6	8	
		1×10^5	11	
		1×10^4	12	
		1×10^3	16	5 HOURS
		1×10^2	16	
		1×10^1	16	
MYCOSEL BROTH	CANDIDA ALBICANS	1×10^5	11	
		1×10^4	14	
		1×10^3	20	
		1×10^2	23	10 HOURS
SYNTHETIC BROTH FOR FUNGI	ASPERGILLUS NIGER (SPORES)	1×10^7	10	10 HOURS
		1×10^6	11	
		1×10^5	16	
		1×10^4	17	
		1×10^3	20	
		1×10^2	22	
		1×10^1	24	23 HOURS
MITIS SALIVARIUS	GAMMA STREPTOCOCCI	1×10^9	3	
		1×10^8	7	
		1×10^7	19	
		1×10^6	19	
		1×10^5	19	
		1×10^4	19	
		1×10^3	19	
		1×10^2	19	
STAPHYLOCOCCUS COAGULASE MEDIUM	STAPHYLOCOCCUS AUREUS	1×10^7	19	
		1×10^4		4.5 HOURS
	STAPHYLOCOCCUS EPIDERMIDIS	1×10^8	19	
		1×10^7	19	
UREASE MEDIUM	PROTEUS MIRABILIS	1×10^7	6.5	
		1×10^6	9	
		1×10^5	16	
		1×10^4	16	
		1×10^3	16	6 HOURS

a) Streptococcus beta hemolytic.

b) Diplococcus pneumoniae.

c) Salmonella typhosa.

d) Neisseria meningitidis.

e) Mima polymorpha.

f) Herellea species.

Each of the most successful culture media we developed to date employs a culture broth chemical gain precipitation type indicator. The growing organisms produce a light absorbing medium precipitate long before the organism cell mass could produce an equal light transmission reduction. This effect is termed chemical gain. The synthesis of light absorbing precipitates from non-absorbing precursors produces a pronounced light transmission reduction through the MLM system, and aids rapid detection.

Suspended particle agglutination and precipitation cannot be MLM detected or in any "space use" designed densitometer. In null gravity the particles do not settle out, even with agglutination. Particle agglutination usually causes a slight light transmission increase. This minimal effect proceeds slowly enough to be confused with cell mass lysis. Although antibody-antigen reaction specificity was an attractive area of investigation, extensive testing uncovered no adaptable MLM application. We therefore decided that the remaining six organisms listed above would require extensive testing to develop special MLM required selective media. Test results with each organism are described in the next sections.

3.3.5.1 Beta Streptococci Detecting Techniques - For these studies, we obtained six recently isolated type A beta hemolytic streptococci strains from a local hospital. We retyped each in our laboratory to confirm type. In addition we tested a beta hemolytic type G, and two gamma hemolytic streptococci.

Several selective media for streptococci are reported in the literature. The Mitis-salivarius medium previously studied for MLM use is but one example. Mitis-salivarius screening tests and other selective media testing for streptococci established that the presence of human saliva can alter selectivity so that Staphylococcus aureus can also grow in the culture medium.

A series of screening tests produced the following MLM-useful medium which is selective for streptococci (beta and gamma).

Salicin Neomycin Broth for Streptococci

Neomycin	0.015 mg
Trypticase	1.5 g
Phytone	0.5 g
Sodium chloride	0.5 g
Salicin	1.0 g
0.3 ml Potassium tellurite (1.0% stock solution)	
Distilled water	97 ml

pH to 7.3

Filter sterilize.

We developed the above medium in our laboratory and is the result of modifying and combining a variety of reported media and selective chemical agents. In initial testing, thirty-five different microorganism species were used as a medium challenge, as well as natural flora from human throats. Only beta and gamma streptococci are found to grow in the medium.

The further MLM differentiation between beta and gamma streptococci is not particularly difficult if fresh blood cells are available. In spite of the fact that glucose presence is known to interfere with red cell hemolysis, a tryptic soy broth containing glucose will support one percent sheep red blood cell hemolysis. This hemolysis is established photometrically at 665 nanometers. Figure 13 presents a curve family obtained with several hemolytic streptococci strains. We observed beta streptococci hemolysis when sheep red blood cells were added to the salicin neomycin broth.

The data presented in Figure 13 do not establish whether or not beta streptococci can be detected in the presence of gamma streptococci, because all curves are pure culture data. We also did not know if tannic acid preserved red cells could be substituted for fresh blood cells. Figure 14 presents clarifying test data. The curves establish that fresh red blood cells can be used to detect beta hemolysis even in the presence of gamma hemolytic streptococci, and that tanned red blood cells will not accomplish the same end.

FIGURE 13

ALTERATION OF CULTURE MEDIUM BY GROWTH OF VARIOUS STREPTOCOCCI IN TRYPTIC SOY BROTH WITH ONE PERCENT SHEEP RED BLOOD CELLS.
CHANGES IN CULTURE MEDIUM DETECTED BY MEASURING PERCENT LIGHT TRANSMISSION AT 665 NANOMETERS

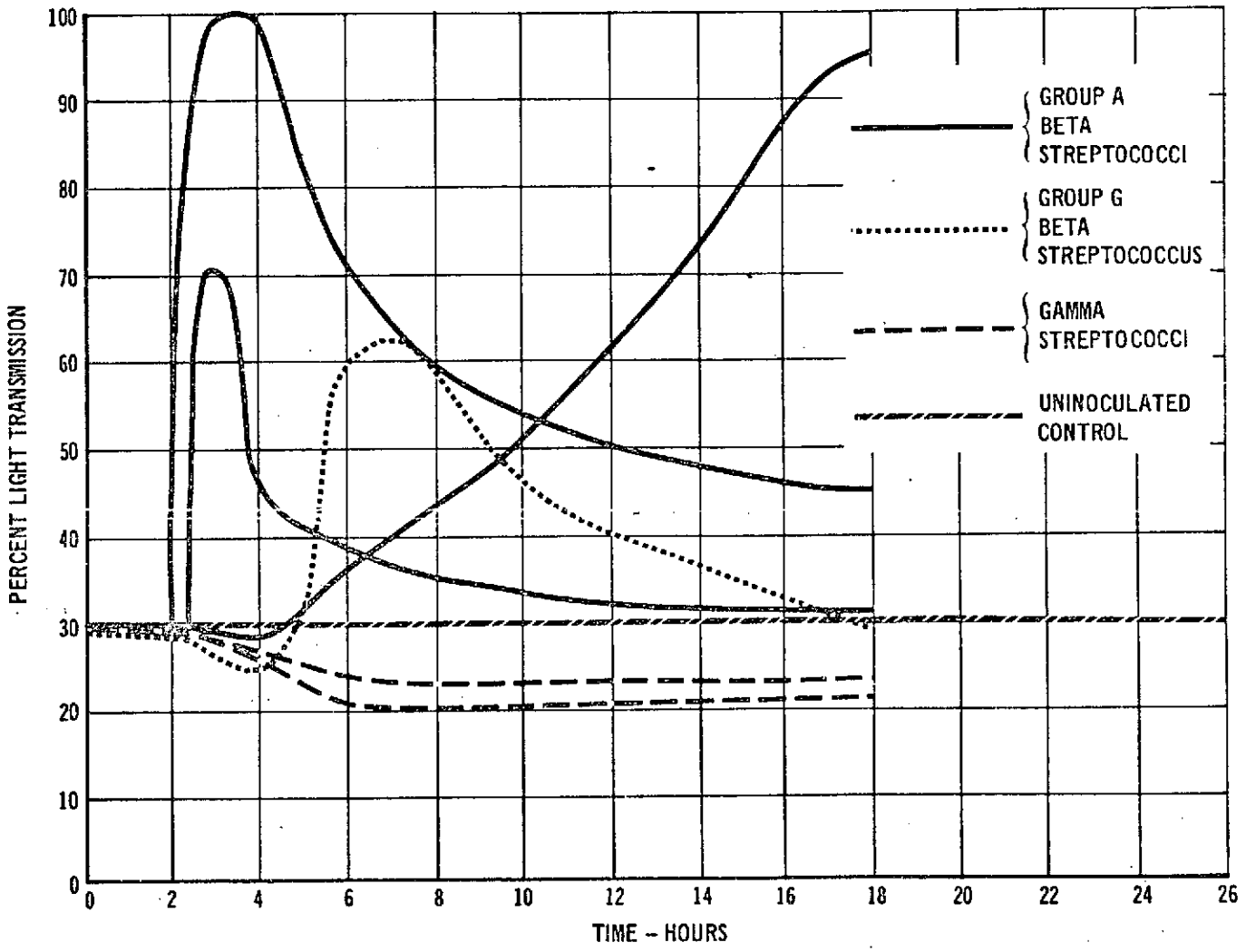
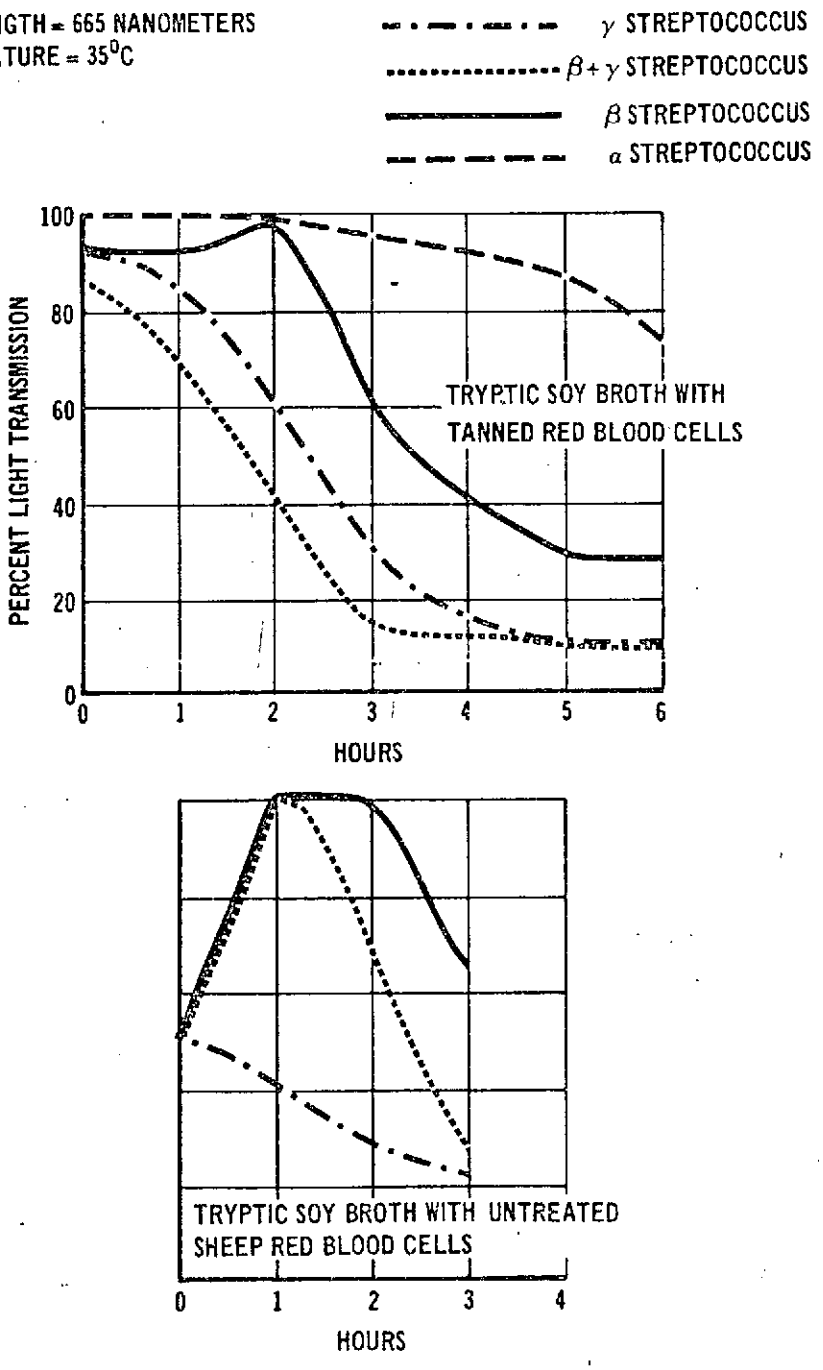


FIGURE 14

COMPARISON OF VARIOUS STREPTOCOCCAL GROWTH PATTERNS IN THE PRESENCE OF TANNED AND UNTREATED SHEEP RED BLOOD CELLS



The restriction to fresh red blood cells severely limits using the technique in MLM applications. Therefore, we sought a stable red blood cell substitute. We tested hemoglobin as a possibility. The investigative results are presented in Figure 15. As depicted by the curves, no unusual characteristic is discernible between hemolytic streptococci types.

As another approach we attempted to take advantage of red blood cell breakdown product differences. We reasoned that beta hemolytic streptococci must produce different intermediate and end products than other hemolytic types. Since we know that violuric acid chelates metal ions, and the resultant color is specific for the type of ion, (cobalt chelates yellow, iron chelates blue), we decided to add violuric acid to hemoglobin in the hope that a blue chelate with hemoglobin iron would be formed as this substance was broken down. Screening tests in broth containing hemoglobin revealed that a blue color and precipitate formed after two to four hours incubation with beta streptococci. Our hopes for using this technique were dashed when subsequent testing revealed one gamma streptococci strain which would remove iron from hemoglobin and produce a blue precipitate faster than beta streptococci. This gamma streptococci strain also attacked violuric acid after 24 hours incubation. We concluded that violuric acid reaction was too sensitive for MLM use since a blue chelate formed without visible hemoglobin destruction.

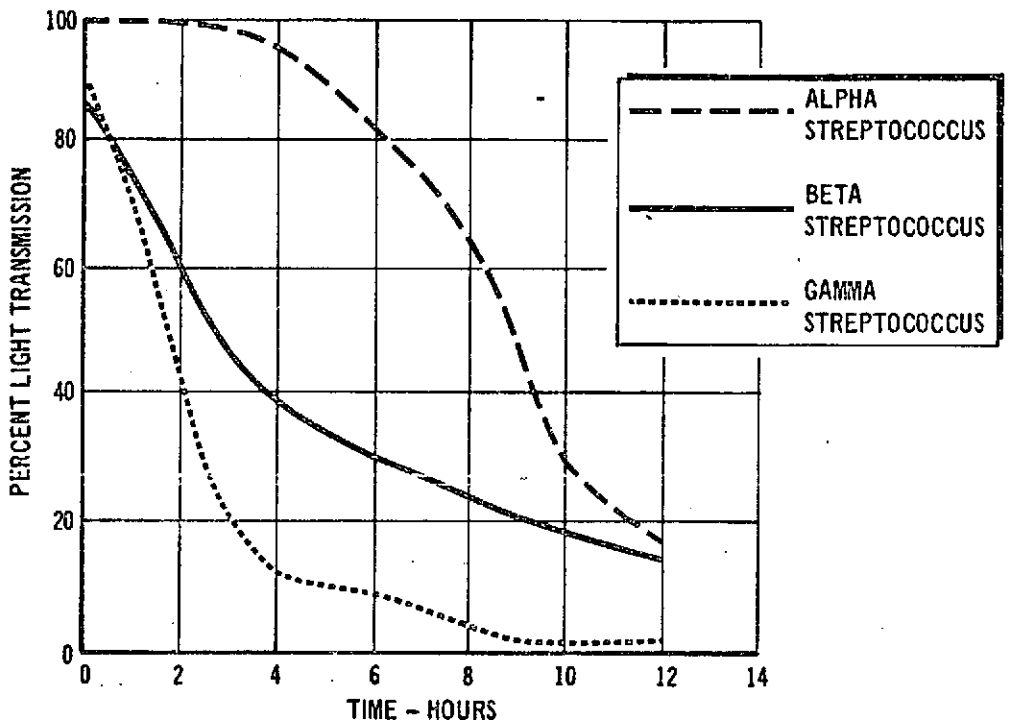
In another approach we attempted to take advantage of the well known type A beta streptococci fibrinolytic activity. Human fibrin clots were reduced to milky opalescence by Branson sonifier treatment. We added sufficient fibrin to tryptic soy broth and produced a milky culture medium. Beta and gamma streptococci were incubated in this broth. Results revealed that pure beta streptococci cultures produced noticeable clearing; however, those cultures containing beta and gamma type mixtures did not clear. We assume that cell mass growth exceeded fibrin destruction.

We made attempts to alter red blood cells so that they could withstand freeze drying, long term storage, and subsequent rehydration.

We obtained partial success by treating 5 ml whole blood with 1 ml of 0.1 percent gluteraldehyde for 2 hours, followed by distilled water washings. Red cells free of surrounding protein are obtained. Such cells could be freeze-dried and rehydrated with culture medium. Unfortunately the results are

FIGURE 15

GROWTH OF VARIOUS HEMOLYTIC TYPES OF STREPTOCOCCI IN CULTURE MEDIUM CONTAINING 0.25 PERCENT HEMOGLOBIN. DETECTION OF LIGHT TRANSMISSION WAS AT 665 NANOMETERS.
INCUBATION TEMPERATURE WAS 35^0 CENTIGRADE



erratic and many repeat tests yielded variable results with beta hemolytic streptococci. Washed red cell gluteraldehyde treatment yielded red cells totally resistant to the streptococci hemolytic action.

Apparently the presence of plasma protein is necessary to prevent too drastic red cell membrane alteration. We obtained partial success with 2-Amino-4-nitrophenol and 2-Amino-2-thiazol followed by ∝-carboxy-o-toluic acid treated red cells which were sometimes attacked by beta hemolytic streptococci. Unfortunately we did not discover the reason for test variability. Lysis occurred spontaneously if the pH was 4.0 or less.

3.3.5.2 Diplococcus pneumoniae Detection Techniques - We obtained success with this organism using serum inulin medium. We found 15.0 percent serum and 0.5 percent inulin in water to be superior to the usual 30 percent serum, literature recommended. Our laboratory tests reveal that even recently isolated D. pneumoniae strains did quite well in this more austere broth, while competing organisms were retarded. Staphylococcus and Nocardia were the most active competitors. Pseudomonas, and Proteus grew to a limited extent, but all organisms tested grew after several days incubation. However, D. pneumoniae grew most rapidly and produced acid from inulin in a few hours. Attempts to add chemical inhibiting agents for other organisms usually inhibited D. pneumoniae to a greater extent. Even potassium tellurite, which could be tolerated to a limited extent in tryptic soy broth, was totally inhibitory to D. pneumoniae at all tested serum inulin water concentrations. MLM tests prove that D. pneumoniae can be successfully grown and detected. Our mixed culture studies reveal that D. pneumoniae can be detected in other organisms' presence under test tube conditions, but a successful acid indicator was desirable in the MLM. We tested congo red, but the serum inulin medium did not produce sufficient acidity to give a rapid precipitate. Other indicators known to be MLM compatible are toxic to D. pneumoniae. Brom thymol blue and phenol red will work for visual screening tests; however, the MLM is almost blind to color changes in these chemicals at concentration unaffecting pneumococci. Greater serum inulin water sensitivty can be obtained by adding iron deoxyribonucleic acid which precipitates at pH values below 7.0 However, iron deoxyribonucleic acid increased competing organism growth rate and negated the sensitivity gained. It is concluded that 15 percent serum with 1 percent inulin is the choice medium D. pneumoniae.

3.3.5.3 Neisseria menigitidis Detection Techniques - N. meningitidis is one of the most fragile pathogens. Tests with this organism are difficult because a high death rate is rapidly achieved; the slowest rate we observed was 3 logs in 8 hours. However, our testing produced a medium for pathogenic Neisseria which has the following composition:

Neisseria Medium

Trypticase	0.75 g
Thiotone	0.75 g
K_2HPO_4	0.4 g
KH_2PO_4	0.1 g
NaCl	0.5 g
Water	85.0 ml
Autoclave above	
Aseptically add 0.25 g hemoglobin in 10 ml sterile water	
1 ml supplement B	
1 ml Colymycin stock =	2500 mcg/100 ml final
1 ml Vancomycin stock =	3000 mcg/100 ml final
1 ml Polymyxin stock =	1500 mcg/100 ml final
0.075 ml Nystatin =	3750 unites/100 ml final
Final pH 7.2	

The above formulation is an MLM compatible broth. Our mixed culture tests established that the medium was totally selective for Neisseria meningitidis and Neisseria gonorrhea with one ntoable exception Four species of Proteus slowly grew in this medium. It is therefore only totally selective in Proteus species absence.

3.3.5.4 Salmonella typhosa Detection Techniques - We reviewed, for possible MLM use, culture media which selectively favor the growth of the Salmonella. They were originally presented in the NASA contract NAS 9-8329 final report, 30 June 1969. That report stated that a medium containing 0.4 percent sodium acid selenite employing thiotone as a nitrogen source would select for salmonellas in 12 to 18 hours, while inhibiting all other organisms.

Our subsequent selenite broth tests revealed that results are variable, and organism strains other than salmonellas which grow well in this broth

have been added to our stock culture collection. These include a Citrobacter strain and E. coli which grow well in selenite presence at pH 7.3

Part of the problem with selenite use to MLM detect salmonellas lies in the choice of growth indication parameters. For example, metabolic activity of large organism quantities, possibly present in the inoculum, produces a culture broth slight brown precipitate. This precipitate is visible to the MLM, and may be interpreted as growth when none is occurring. The sodium acid selenite selective action depends upon a pH near 7.0, and increasing pH results in a toxicity loss for organisms other than Salmonella species. This pH increase occurs when mixed inocula stand in the culture medium for time periods over 12 - 16 hours. The results is a slight turbidity which the human eye can spot as a false positive, but the MLM reads as growth.

3.3.5.5 Herellea-Mima species Detection Techniques - Numerous literature reports establish that these organisms tolerate high pH, have remarkable lipolytic powers, and are able to use various fats as a sole carbon source. Unfortunately, none of these tests proved very useful in our selective medium development. Existing Herellea culture media were proven MLM worthless.

Our attempt to capitalize on high pH tolerance results in a culture medium of pH 10.5 in which Nocardia corallina and one strain of Escherichia coli grew faster than Herellea. We discovered that while Herellea tolerates two percent Tween 80 and pH 10.5, so does E. coli, N. corallina and a host of naturally occurring contaminants.

After many frustrating months with the Herellea-Mima group, our consensus is that the following medium is superior to all published previously, and although not absolutely selective, Herellea will usually outstrip all competitors and give rapid growth which can be MLM detected.

Herellea Medium

1 g ethanol	0.0 g $MgSO_4$
0.3 g NH_4CL	99 ml H_2O
0.5 g NaCl	pH 6.0
0.1 g K_2HPO_4	0.1 ml Nystatin (5000 units)

The above medium was selective when inoculated with 40 different stock culture organism species:

The choice chemical selective medium we developed for S. typhosa employs a precipitation type indicator and is formulated as follows:

Salmonella typhosa medium

Sodium acid selenite	0.5%
Dulcitol	1.0%
Thiotone	0.5%
Sodium Chloride	0.5%
Phosphate buffer (KH_2PO_4)	0.1%
Ethyl alcohol	2.0% vol./vol.
pH 6.8, filter sterilize	

This formulation permits the rapid Salmonella typhosa growth and a heavy precipitate of sodium desoxycholate. Other salmonellas tested do not grow. These include S. enteriditis, Salmonella para A and para B, S. schottmulleri and S. typhimurium.

The two percent ethanol requirement creates a disadvantageous storage problem with S. typhosa medium. It is obvious it cannot be freeze-dried for sample holder placement.

A freeze drying - compatible medium capable of selecting S. typhosa and a few other Salmonellae is reported later.

The Mima selective medium has the following formulation:

Mima Medium

4% Tween 40	Mix first
.006% Nile Blue A	
4% NaCl	
.05% Yeast extract	
0.3% K_2HPO_4	
pH 8.9	
0.5% NH_4CL	

Mixed culture difficulties will arise unless the medium is prepared in the sequence described above. If a slight precipitate forms, the medium

has been improperly prepared. Normal color is a light purple with no precipitate. Improperly prepared medium will permit Pseudomonas species growth, however properly prepared media is quite selective for Mima.

3.3.6 Mixed Culture Studies Evaluation and Modification - It was apparent early that a great possible advantage lay in preprogramming the detection chambers with dried or semi-dried culture media. This technique, if successful, will obviate all the previously encountered problems of stored media water loss. Sterility maintenance is easier with dried media.

When we developed approximately half the required selective media for the MLM, we began studies with freeze-dried media and rehydrated media. Our purpose was demonstrating freeze-dried media selectivity retention present prior to the freeze-drying process.

Preliminary test data are presented in Table 3. These data indicate that five minutes diluent fluid contact yields culture media sufficiently similar to controls and yields similar MLM growth curves. We interpret this to mean that the media freeze dried selective agents redissolve in sufficient propertions and yield a satisfactory medium. In examining the Mannitol Salt medium it is noted that 30 seconds diluent contact produced faster microbial growth than the control. Pragmatically, the results for each rehydration period are quite variable. This is because the dried salts and proteins redissolve slowly. However, a five minute waiting period is quite MLM compatible.

After we developed most of the selective MLM media required, we performed extensive testing with freeze-dried media rehydrated with fluid containing clinical throat, urine, and fecal specimens.

We deemed it probable that problems would arise with rehydrated freeze-dried media containing urine, feces, throat, and skin sample substances. Therefore, we performed tests to determine such problems' extent.

MLM culture media selectivity, in some cases depends upon initial medium pH. Other media require a pH change to produce a positive reaction. We felt it imperative to determine the diluted urine and feces effect upon MLM culture medium pH.

Each test broth was frozen in 5 ml aliquots for these studies. After drying, each aliquot yield was mixed well, weighed, and 5 equal amounts

TABLE 3
COMPARISON OF GROWTH DETECTED BY MLM IN TESTS WITH FRESH AND REHYDRATED FREEZE DRIED MEDIA

CULTURE MEDIA WAS FREEZE DRIED ON FILTER PAPER. REHYDRATION WAS ACCOMPLISHED BY CONTACT WITH DISTILLED WATER FOR LENGTHS OF TIME VARYING FROM 30 SECOND TO 5 MINUTES. WATER VOLUME EQUAL TO ORIGINAL MEDIA VOLUME.

		TIME IN HOURS FROM INOCULATION TO FULL SCALE DETECTION BY MLM				
TEST ORGANISM	CULTURE MEDIUM	30 SECOND REHYDRATION	2 MINUTE REHYDRATION	3½ MINUTE REHYDRATION	5 MINUTE REHYDRATION	CONTROL (FRESH)
PSUEDOMONAS AERUGINOSA	CETRIMIDE	15	8¾	8½	8½	7
CANDIDA ALBICANS	MYCOSEL	18	14½	18	13	12
ESCHERICHIA COLI	COLIFORM	–	3½	2	4½	5½
PROTEUS MIRABILIS	UREASE	6½	5¾	5¾	5¾	4¾
ASPERGILLUS NIGER	SYNTHETIC FOR FUNGI	–	> 20	> 20	16¾	13½
STAPHYLO-COCCUS AUREUS	MANNITOL SALT	5½	9¾	6	8¾	10

distributed into tubes so that each tube would be rehydrated with 1 ml liquid.

We tested the following MLM media: Coliform, Cetrimide, Salmonella, Salicin Neomycin, Staphylococcus, Synthetic for Fungi, Urease, and Mycosel. We did not test Herellea broth, as it cannot be freeze-dried.

Aliquots of sterile urine with adjusted pH values ranging from 4.5 - 9.0 were diluted 1:10 with sterile, pH 7.0 distilled water. We recorded the resulting dilution pH values. We added one ml of each pH dilution to each prepared freeze-dried medium aliquots. Each mediums' pH values were recorded at approximately one minute following rehydration.

All media rehydrated well, with initial turbidity completely dissolved in one minute, all cases. Resulting prepared media pH effects are presented in Table 4.

Data inspection reveals all final pH measurements within acceptable limits.

We repeated the above tests with more concentrated urine samples, with urine/water ratios ranging from 1:2 to 1:4. Table 5 reveals a few undesirable final pH measurements for fungi detection media formulations.

Pea-sized fecal material amounts were mixed well with 10 ml, pH 7.0 distilled water. We added one ml to the freeze-dried media prepared as (loc cit 3.3.1). We then recorded the pH's of the rehydrated media. Our results are in Table 6. All final pH measurements are within acceptable limits.

3.3.7 Methods Development for Determining Selective Microorganism Numbers - In the early MLM developmental stages it was hoped that growth rate differences could yield useful information re' relative organism numbers present in the inoculum. Mixed culture studies soon demonstrated that such an approach was impractical with mixed populations. We designed and evaluated other approaches.

Our subsequently evolved approach as the choice method employed filtration bed series'. We originally employed cellulose, glass, and asbestos fibers. We also tested alumina.

Cellulose fiber test results are presented in Table 7.

Bacteria migrated 1.0 cm in 10 minutes with at least a two log population

TABLE 4
FINAL pH OF MEDIA REHYDRATED WITH HUMAN URINE AND WATER

	pH OF MEDIA PRIOR TO FREEZE-DRYING	pH OF DILUTED URINE USED TO REHYDRATE MEDIA							CONTROLS REHYDRATED WITH H_2O AT pH 7.0
		A pH 4.5	B pH 5.0	C pH 6.0	D pH 7.0	E pH 7.5	F pH 8.0	G pH 9.0	
		RESULTANT pH AFTER REHYDRATION							
COLIFORM (NONBUFFERED)	PH 7.4	7.0	7.05	7.15	7.3	7.35	7.35	7.45	7.3
CETRIMIDE	7.5	7.2	7.25	7.29	7.32	7.32	7.3	7.32	7.3
SALMONELLA (BUFFERED)	6.7	6.5	6.5	6.5	6.52	6.52	6.59	6.6	6.52
BETA STREPTOCOCCUS (NONBUFFERED)	7.3	7.15	7.25	7.3	7.35	7.4,	7.4	7.42	7.4
STAPHYLOCOCCUS	7.2	6.2	6.25	6.3	6.5	6.7	6.8	7.0	6.5
SYNTHETIC FUNGI (BUFFERED)	7.3	6.4	6.6	6.8	7.0	7.05	7.1	7.1	6.95
CANDIDA	6.9	6.5	6.5	6.6	6.6	6.65	6.65	6.75	6.55
UREASE	6.8	6.4	6.4	6.45	6.5	6.5	6.55	6.55	6.5

TABLE 5
FINAL pH OF CULTURE MEDIA AFTER REHYDRATING WITH DILUTED URINE

URINE/H_2O RATIO	URINE pH	COLIFORM OPTIMUM pH 7.4		SALMONELLA OPTIMUM pH 6.7		SYNTHETIC FOR FUNGI OPTIMUM pH 7.3		CETRIMIDE OPTIMUM pH 7.5	BETA STREPTO-COCCUS OPTIMUM pH 7.5	CANDIDA OPTIMUM pH = 6.9
		NON-BUFFERED	BUFFERED	NON-BUFFERED	BUFFERED	NON-BUFFERED	BUFFERED	NON-BUFFERED	BUFFERED	BUFFERED
1:2	6.85	7.25	7.20	6.65	6.7	–	–	7.4	7.45	6.9
1:3	6.85	7.35	7.3	6.55	6.6	7.2	–	7.45	7.5	6.7
1:3	4.5	6.7	7.1	6.7	6.8	*5.3	–	7.2	7.0	*5.7
1:3	9.0	7.7	7.4	–	–	–	–	7.6	7.65	7.2
1:4	6.9	7.35	7.25	6.5	6.6	7.3	6.9	7.45	7.5	6.65
1:4	9	7.7	7.4	6.0	6.6	7.9	7.75	7.6	7.65	7.2
1:4	4.5	7.0	7.1	6.8	6.9	*5.5	*6.0	7.3	7.1	*5.6
H_2O 100%	7.0	7.4	7.4	6.7	6.7	7.3	7.3	7.5	7.5	6.9

*UNDESIRABLE PH FOLLOWING REHYDRATION

TABLE 6

STUDIES OF FREEZE DRIED MEDIA REHYDRATED WITH HUMAN FECES AND WATER

MLM CULTURE MEDIA	pH BEFORE FREEZE DRY (MEDIUM)	pH AFTER REHYDRATION WITH FECAL SAMPLE & H_2O	
		TRIAL 1	TRIAL 2
COLIFORM	7.4	7.2	7.3
CETRIMIDE	7.5	7.3	7.3
SALMONELLA	6.7	6.6	6.5
SALICIN-NEOMYCIN	7.3	7.3	7.25
STAPHYLOCOCCUS	7.2	6.8	6.9
SYNTHETIC- FUNGI	7.3	7.1	7.15
MYCOSEL	6.9	6.65	6.7
UREASE	6.8	6.55	6.4
DISTILLED H_2O & SAMPLE		6.8	7.0

number reduction. The physically much larger yeast cells yielded less reproducible results, but this problem is probably a filter length function.

We studied the filter system bacterial migration time relationships. Table 8 presents our data. The results indicate that between 5 and 10 minutes difference in migration is not great. At 15 minutes there is significantly greater migration than that observed at 5 minutes. Additional asbestos fiber tests established that they are superior to other fibers tested. In addition to greater reproducibility there is greater handling ease because asbestos fibers packed into filtration beds did not tend to swell and change shape as did cellulose and alumina. Glass fibers gave erratic results. Asbestos fiber test results are presented in Table 9. It is apparent that these filters effectiviely remove living organisms passing through them, and within acceptable reproducibility limits.

This section and section 3.5 describe our developments paving the way for the automatic sampling device design for liquids and semi-solids which the following section describes in greater detail.

It is sufficient at this stage to point out the basic assumption re' determining relative organism numbers present in sampled material resembles the "most probable number" methods used in determining water or sewage bacterial counts. However, replacing dilution blanks, a single passageway filtration bed series accomplishes clarification and serial dilution. Incorporating freeze-dried selective media between filters, organism growth in each serial dilution chamber is obtained. Filtration bed thickness preadjustment reduces one to two logs the microorganism population present per unit fluid volume. Therefore, if filtration beds removing one log population per unit volume are used, and six serial dilutions are made, yielding only first three chambers' growth, we determine there were originally more than 1,000 and less then 10,000 organisms present.

3.4 Detector Development

We present a laboratory model MLM description under section 3.3.2, Laboratory MLM Instrument. During the first contract quarter, we made the decision to concentrate on 665 nm wavelength detection units because our results are much better than with 900 nm detection units. Our previously reported difficulties

TABLE 7
MIGRATION OF FOUR SPECIES OF ORGANISMS ON WHATMAN FILTER PAPER NUMBER 541
Migration time was 10 minutes

ORGANISM NAME	NUMBER OF ORGANISMS INOCULATED ON FILTER	NUMBER OF ORGANISMS MIGRATING 1.0 CM		
		TRIAL 1	TRIAL 2	TRIAL 3
ESCHERICHIA COLI	2.7×10^4	7.2×10^2	4.0×10^2	8.5×10^2
BACILLUS SUBTILIS (SPORES)	1.8×10^4	5.3×10^2	1.1×10^3	4.1×10^2
STAPHYLOCOCCUS AUE	1.0×10^5	3.0×10^2	9.2×10^2	4.7×10^2
SACCHAROMYCES CEREVISIAE	6.0×10^3	4.8×10^2	1×10^1	0

TABLE 8
EFFECT OF TIME ON BACTERIAL MIGRATION THROUGH FILTER PAPER

1.4×10^7 ESCHERICHIA COLI PLACED ON 0.5 x 1.5 CM FILTER PAPER.
PAPER WAS POSITIONED VERTICALLY AND THE BASE WAS ALLOWED TO CONTACT A WATER RESERVOIR.
MICROBIOLOGICAL ASSAY WAS MADE OF THE TOP 0.5 M OF PAPER AFTER APPROPRIATE TIMES.

TYPE OF FILTER	LENGTH	TIME IN MINUTES	NUMBER OF ORGANISMS MIGRATING 1 CM
WHATMAN NO. 1	1.0 CM	5	1.2×10^2
		5	4.5×10^2
		6	2.7×10^2
		7	2.0×10^2
		10	7.8×10^2
WHATMAN NO. 541	1.0 CM	5	1.0×10^1
		5	8.0×10^1
		6	5.0×10^1
		7	1.5×10^2
		14	8.0×10^1
		15	1.5×10^2

TABLE 9
RESULTS OF USING ASBESTOS FIBERS TO DILUTE SUSPENSIONS OF MICROORGANISMS

FILTER BEDS FOR THESE TESTS WERE 1 MM IN DIAMETER. A VACUUM EQUIVALENT TO 10 INCHES OF WATER WAS USED TO AID FILTRATION. TEST ORGANISMS WERE ESCHERICHIA COLI AND CANDIDA ALBICANS.

ORGANISM AND NUMBER	LENGTH OF FILTER BED	AMOUNT OF FLUID	MICROBIOLOGICAL ASSAY RESULTS AFTER FILTRATION	DILUTION ACCOMPLISHED
ESCHERICHIA COLI 8.0×10^5 TOTAL	1 MM	1 λ	REPLICATE TRIALS 8.8×10^3 1.1×10^3	APPROX 2 LOGS
ESCHERICHIA COLI 8.0×10^5 TOTAL	2 MM	5 λ	REPLICATE TRIALS 4.4×10^2 1.4×10^2	APPROX 3 LOGS
ESCHERICHIA COLI 8.0×10^5 TOTAL	3 MM	5 λ	REPLICATE TRIALS 8.0×10^1 8.0×10^1	APPROX 4 LOGS
CANDIDA ALBICANS 4.5×10^5 TOTAL	0.5 MM	10 λ	REPLICATE TRIALS 3.0×10^4, 1.5×10^3 2.0×10^4	APPROX 1 LOG
CANDIDA ALBICANS 4.5×10^5 TOTAL	1.0 MM	10 λ	REPLICATE TRIALS 1.4×10^3, 8.0×10^2 1.0×10^3	APPROX 2 LOGS
CANDIDA ALBICANS 4.5×10^5 TOTAL	2.0 MM	10 λ	REPLICATE TRIALS 2.0×10^1, 1.0×10^1 4.0×10^1	APPROX 4 LOGS

with 665 nm detectors were due to insufficiently developed 665 nm solid state emitters. Early in the present contract suitable 665 nm emitting diodes became readily available. We made several tests quantitatively evaluating the MLM optical section performance, using emitting and detecting diodes presently or previously employed. The detector is the light sensitive diode HPA 4203, and the emitters are MV10B3, emitting at 665 nanometers (nm) wavelength, and HPA 4120 emitting at 900 nm. All tests measured the detecting diode incident power while controlling the following parameters:

- Emitter drive current.
- Emitter - detector distance.
- Emitter and detectors' central axis angular offset

In our first test, we aligned the emitting and detecting diodes' central axes and set their faces 0.1 inches apart. We varied the emitter drive current from 7 to 70 milliampers, while recording the detector photocurrent output. Figure 16 shows the system using the MV10B3 to be somewhat more powerful than the HPA 4120, although both show approximately the same slope. Subsequent MV10B3 testing shows a wide variation in detected light output versus drive current. This is believed due to emitter chip variations, and the shape and quality of the epoxy lens.

We developed a general expression showing the functional relation between incident power, drive current, and distance. It follows:

$$(1) \qquad I_p = \frac{aI_D}{(d_o + d)^2}$$

I_p = Photocurrent

I_D = Drive current

We drive the test emitters at a 50 milliampere direct current and recorded the photocurrent while varying distances between emitter and detector zero to 0.8 inches. Using the data points to evaluate the constants a and d_o a first approximation follows (MV10B3):

$$(2) \qquad I_p = \frac{6 \times 10^{-4}}{(0.15 + d)^2}$$

I_D = microamps

I_D = Drive Current, ma

Figure 17 plots Equation 2 and a similar one for the HPA 4120 and shows good experimental data agreements. Equation 2 is valid for air medium between emitter

FIGURE 16
TRANSMITTANCE VS DRIVE CURRENT

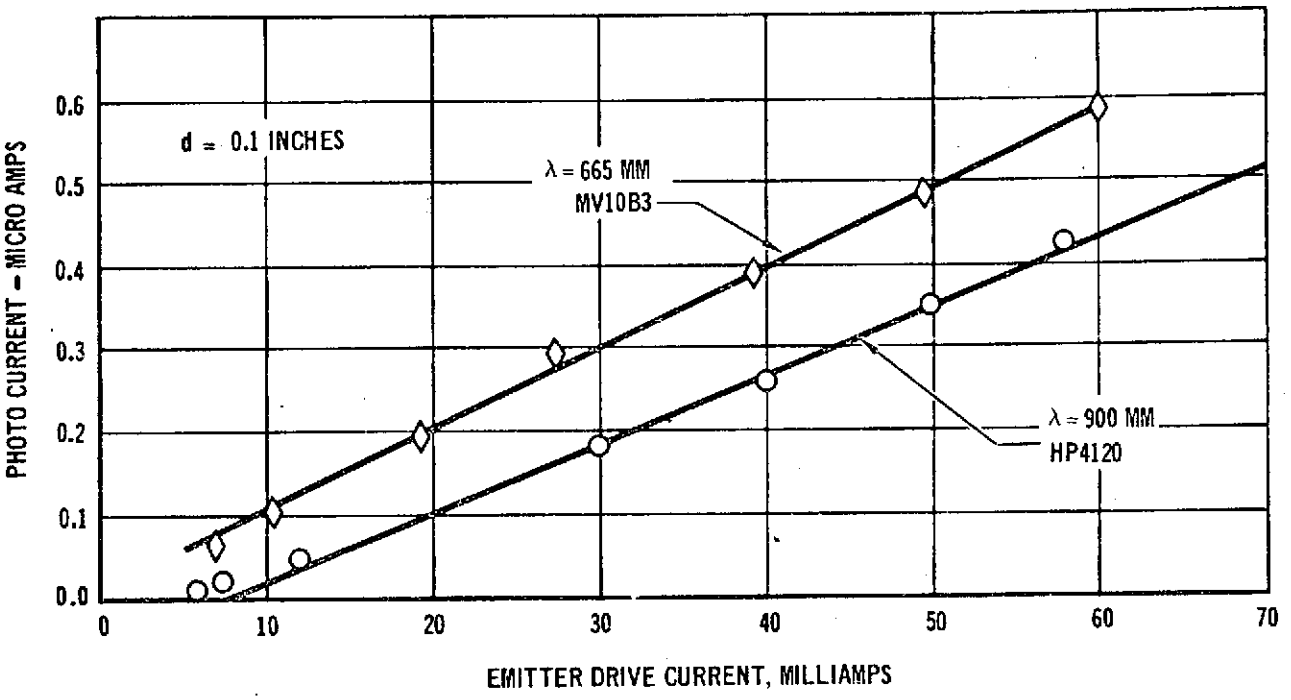
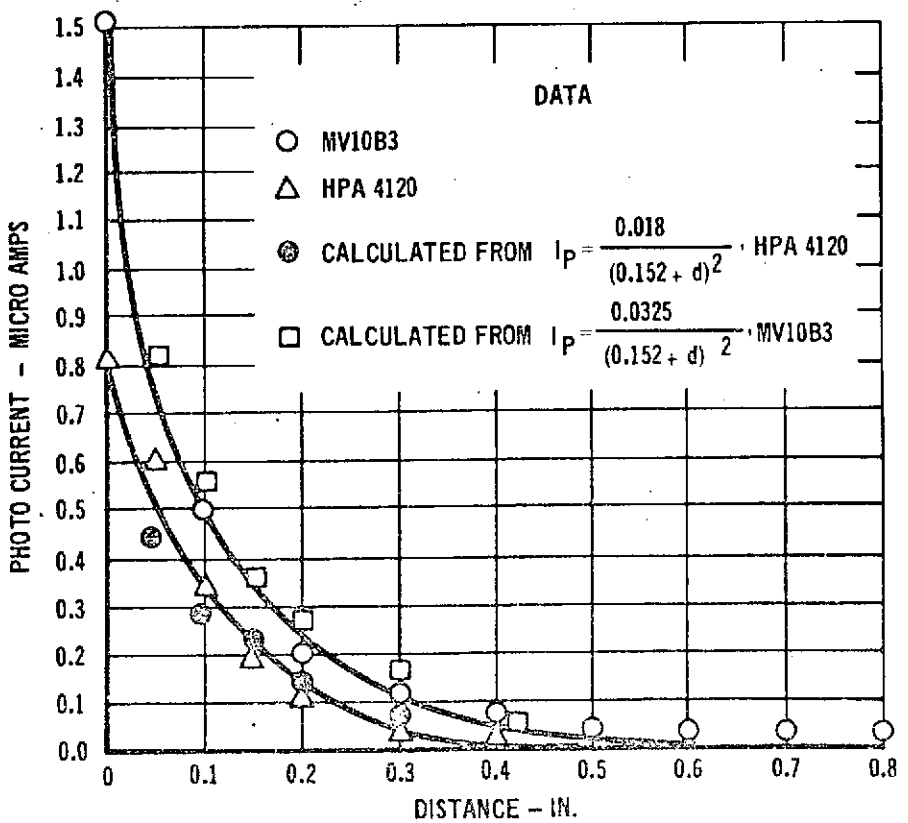


FIGURE 17
TRANSMITTANCE VS DISTANCE



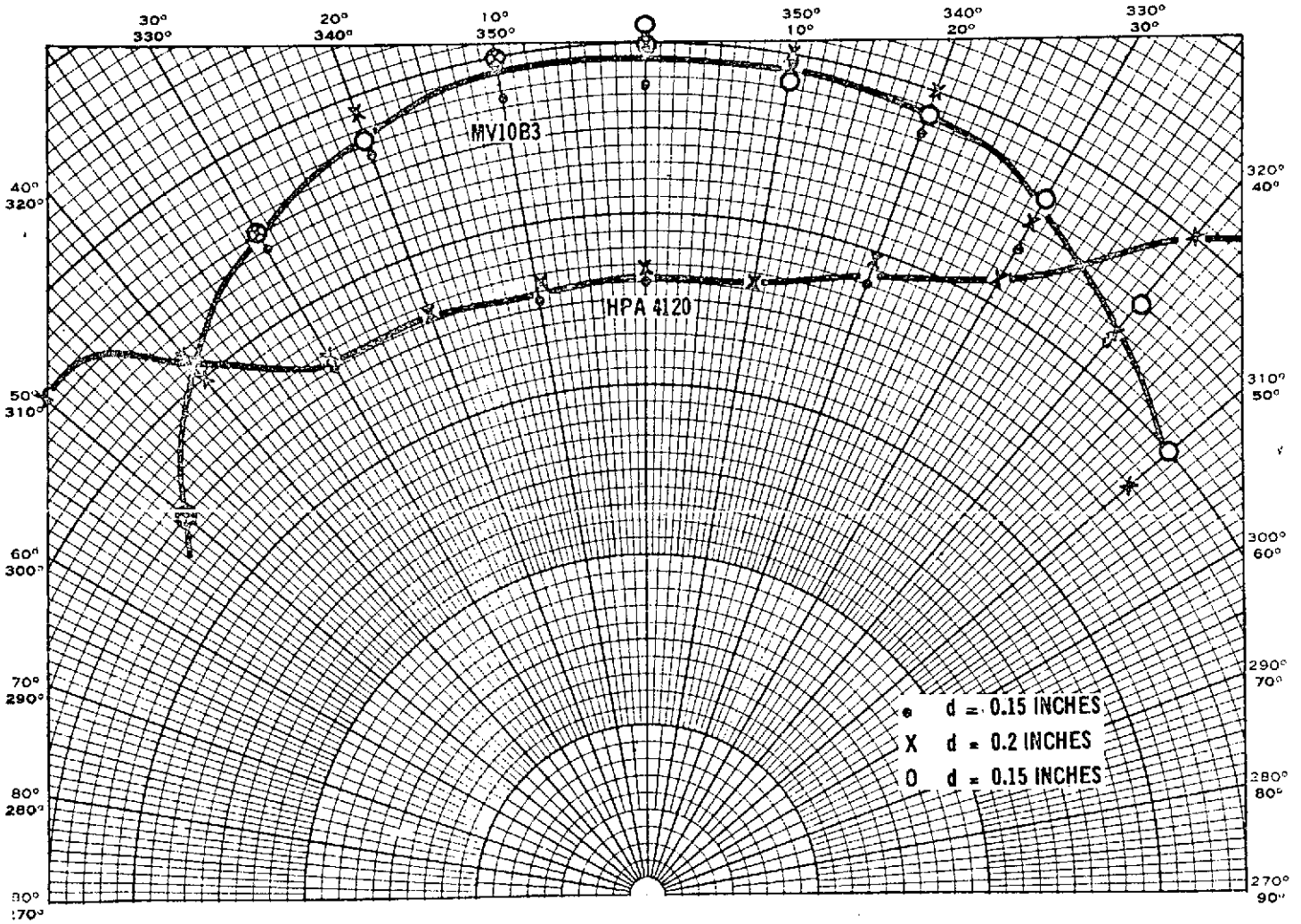
and detector and for axial alignment of the faces. However, the functional dependence on distance and drive current is invariant.

We evaluated the angular dependence of the emitting and detecting diodes, in the third test of this series. A turntable was set up so that the angles between the diodes' central axes could be measured. The emitter drive was set at 50 milliamperes and the percent transmittance (referenced to received signal at zero degrees) was calculated for the angle range indicated in Figure 18. Trials for both emitters were run with detector-emitter distances of 0.15 and 0.2 inches.

The polar graph of Figure 18 shows that the beam widths for both emitters are broader than those given in the manufacturers data sheets. The HPA 4120 has side lobse somewhat more powerful than the main lobe. The beam pattern obtained with the MV10B3 shows a flat response over an angle of $\pm$ 10 degrees, with transmittance down five percent at $\pm$ 20 degrees. Such a pattern shows that slight misalignments, with this pattern, will have less effect on the power incident on the cell center than that experienced with the HPA 4120 (IR) diodes. However, the broad pattern could place a limit on the minimum distance required between adjacent "on" emitters to minimize stray light entering a detector diode.

3.4.1 Results of Optical Tests - As a result of these tests and analyses, it was concluded that the new solid state 665 nm emitter diode (MV10B3) or equivalent can be directly substituted into the existing MLM without degradation of system performance. A resulting improvement in detectability of color change and precipitates will thereby enhance detection time.

FIGURE 18
PERCENT TRANSMITTANCE VS ANGLE



3.4.2 Design and Fabrication of Sampling Device - Any MLM sampling unit used in spaceflight conditions will be required to perform several operations automatically. Figure 19 illustrates the throat culture procedures required and similar steps are required for feces, environmental surfaces, human skin, and urine samples. Early null g compatible MLM designs entailed two parallel efforts.

3.4.2.1 Rotating Disc Design - The first design was based upon Reference 10 which reports the advantages provided by using a rotating disc, thus achieving sample transfer and bubble control. Figure 20 illustrates the device.

We designed the disc for solid and liquid sampling utilizing a ring of detection ports for different media and sample concentrations. These ports are fed by a series of processing chambers. A sample was injected via septum into a central reservoir where initial filtration took place. Disc spinning moved the sample peripherally by variable centrifugal force. After passing through the filter, passageways under the film permitted fluid movement into a ring of concentration chambers where sample fluid was absorbed. Different concentrations were achieved by varying the chamber absorbption. If concentration was not necessary, these chambers were omitted (or free of absorbent material). At a specified fluid pressure resulting from disc spinning the sample broke the frangible seal barring the media reservoirs. After media reservoir mixing the pressure was raised higher, breaking the next frangible seals barring detection port access. The sample fluid and media mixture then flowed into detection port and back under the bottom cover film overflow passages until the fluid distance along the disc was the same in both filling and overflow passages. The displaced air returned to the disc center. The disc was then decelerated to a minimum angular velocity, keeping any bubbles concentrated along one detection port side and beyond the light path. Growth detection took place with one detection station while the disc was spinning. If dilution was required, the first stage was syringe diluted before filling the disc. The second dilution stage was accomplished by enlarging the media reservoirs.

The disc was heat formed, high density polyethylene. Each face cover was a clear mylar-polyethylene laminate which was heat sealed to the disc

FIGURE 19
PROPOSED OPERATION OF SAMPLING UNIT FOR THROAT EXAMINATION

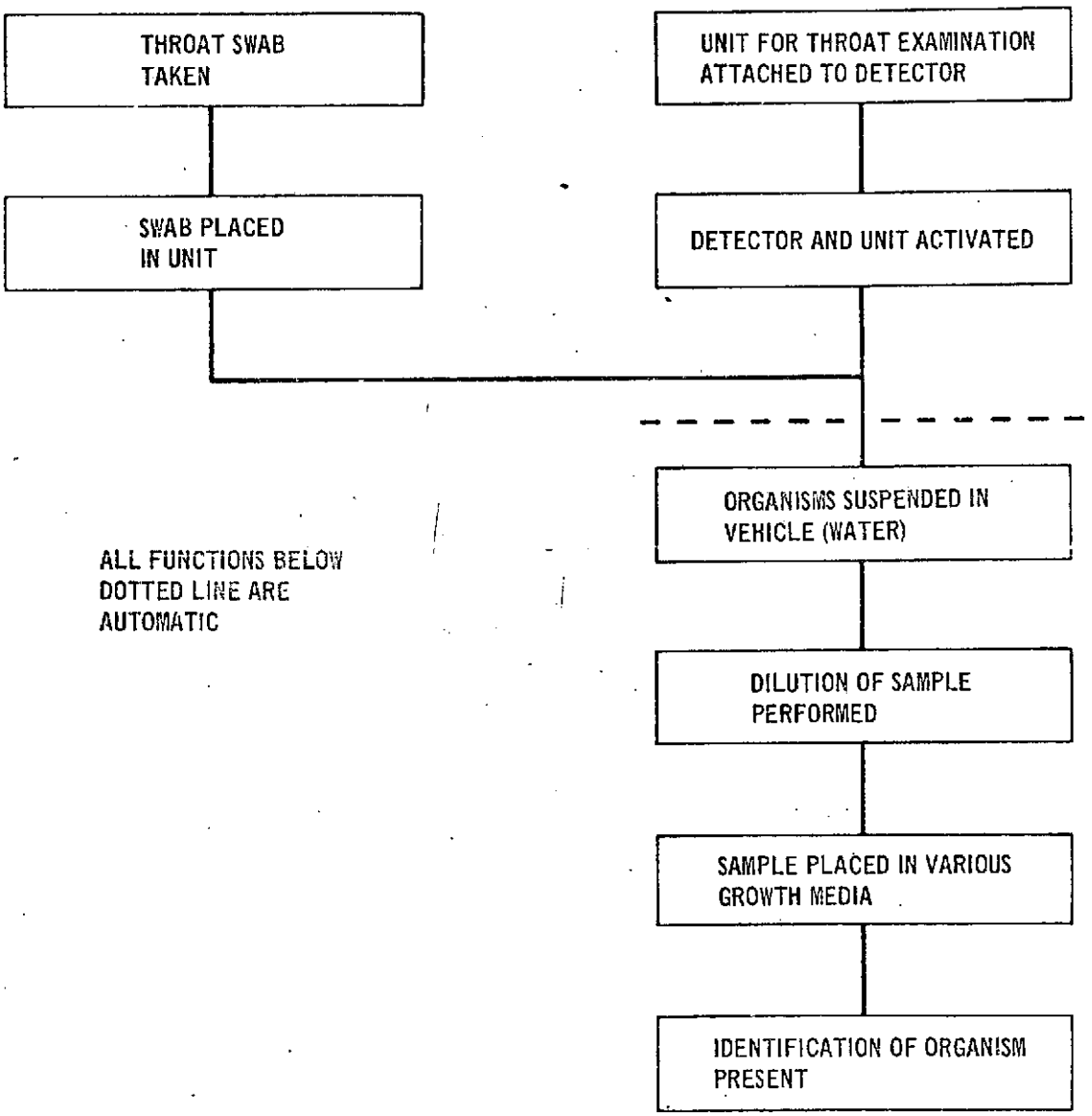
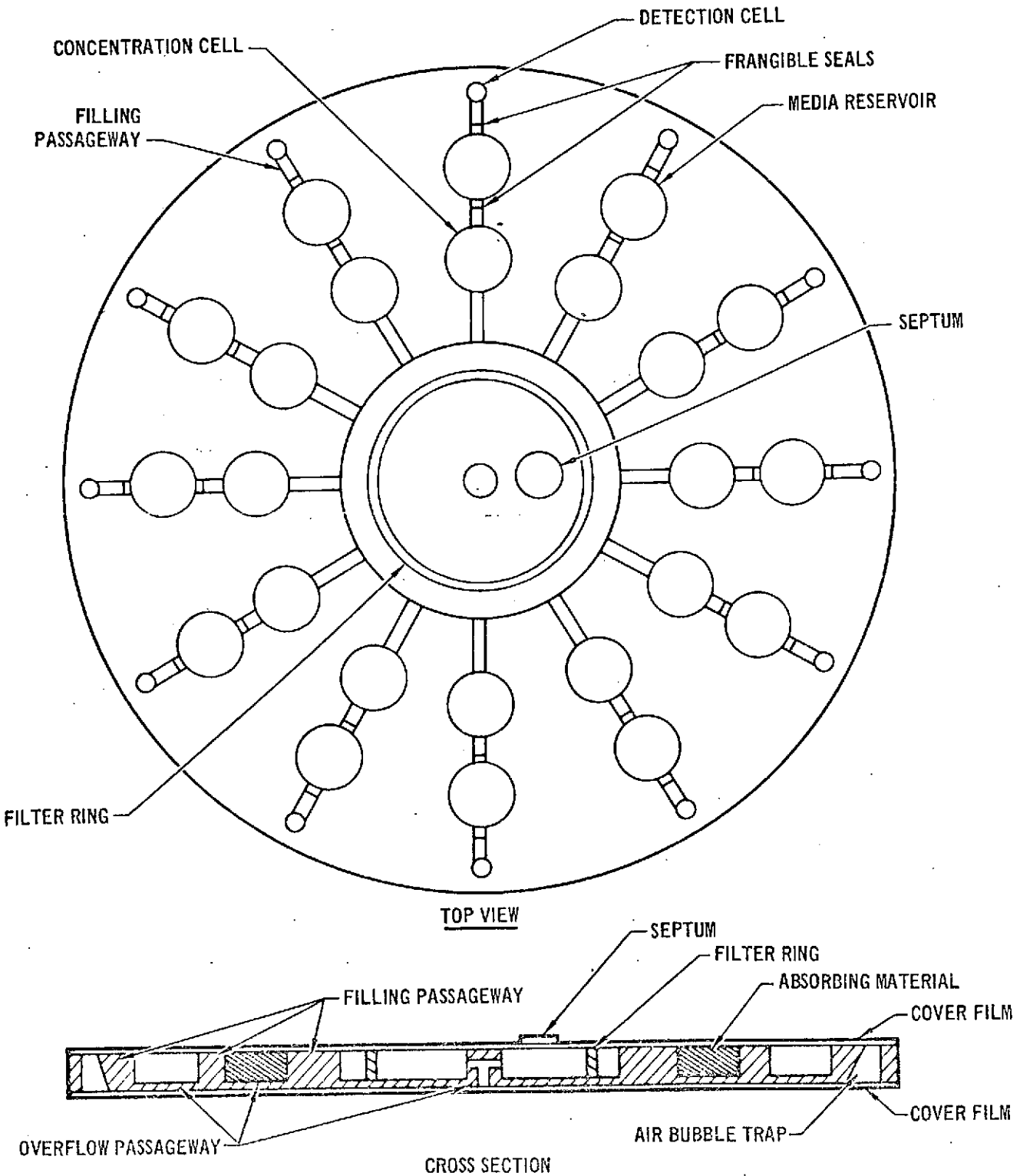


FIGURE 20
ROTATING DETECTION CELL CONCEPT



surrounding the various chambers. Leaving the film unsealed formed the passageways between chambers in these areas. The frangible seals were formed by carefully controlling the point sealing and thus providing a low strength bond. The disc and cover materials were selected for their excellent moisture barrier properties and fabrication ease.

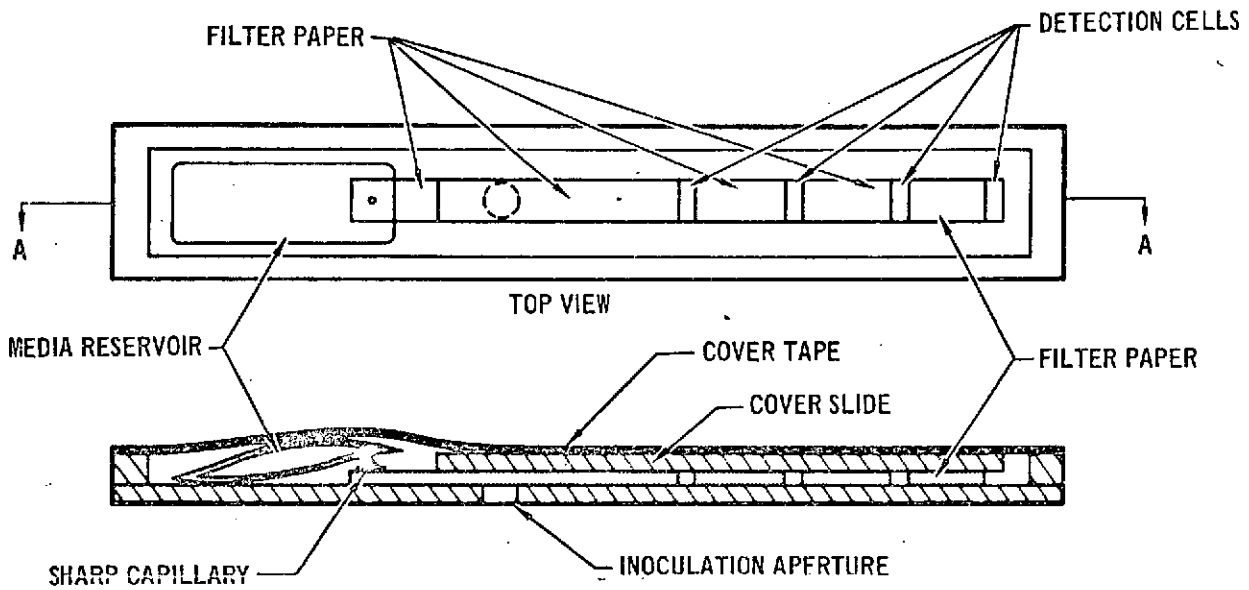
The principal disc device advantage was that the same sample could be applied to several media simultaneously, and at different sample concentrations, without extra handling. Rotating the disc reduced the number of detection stations to one per disc, but, this correspondingly increased the electronic complexity. The major disc advantage was that centrifugal force could keep bubbles out of the viewing area.

The major disadvantages were mechanical complexity and the need to fluid-suspend solid samples before disc filling. The frangible seals were also difficult to form and therefore not sufficiently reliable.

3.4.2.2 Filter Device Design - The second automatic sampling unit design is called a filter device. The action mode is capillary fill through either filter paper or glass fiber filter beds. Figure 21 illustrates the device's top and side view. We tested the filter approach with microorganisms and results exceeded expectations. We have made several modifications since our original designs and we have made rapid enough progress that breadboard units are now ready for MLM testing.

In descriptive detail, the filter device originally consisted of filter material strips in series and sandwiched between two glass or clear plastic slides. The sample was swab or probe applied to the filter material through a bottom slide inoculation aperture. Teflon adhesive tape sealed the aperture. The media reservoir was a small mylar-polyethylene laminate bag positioned over a sharpened capillary attached to the filter material. When the cover film is pressed onto the reservoir, the capillary punctures the bag. The microbe carrying fluid, thus released, advances through the filter. In minutes fluid fills the gaps between the filter paper pieces. Growth detection takes place in the gaps. Particulate matter is quickly filtered out of the sample and the microbes are distributed in a diminishing concentration gradient through the filter length. The gaps remain filled for several days.

FIGURE 21
DETECTION CELL CONCEPT USING CAPILLARY ACTION
FOR AUTOMATIC FILL



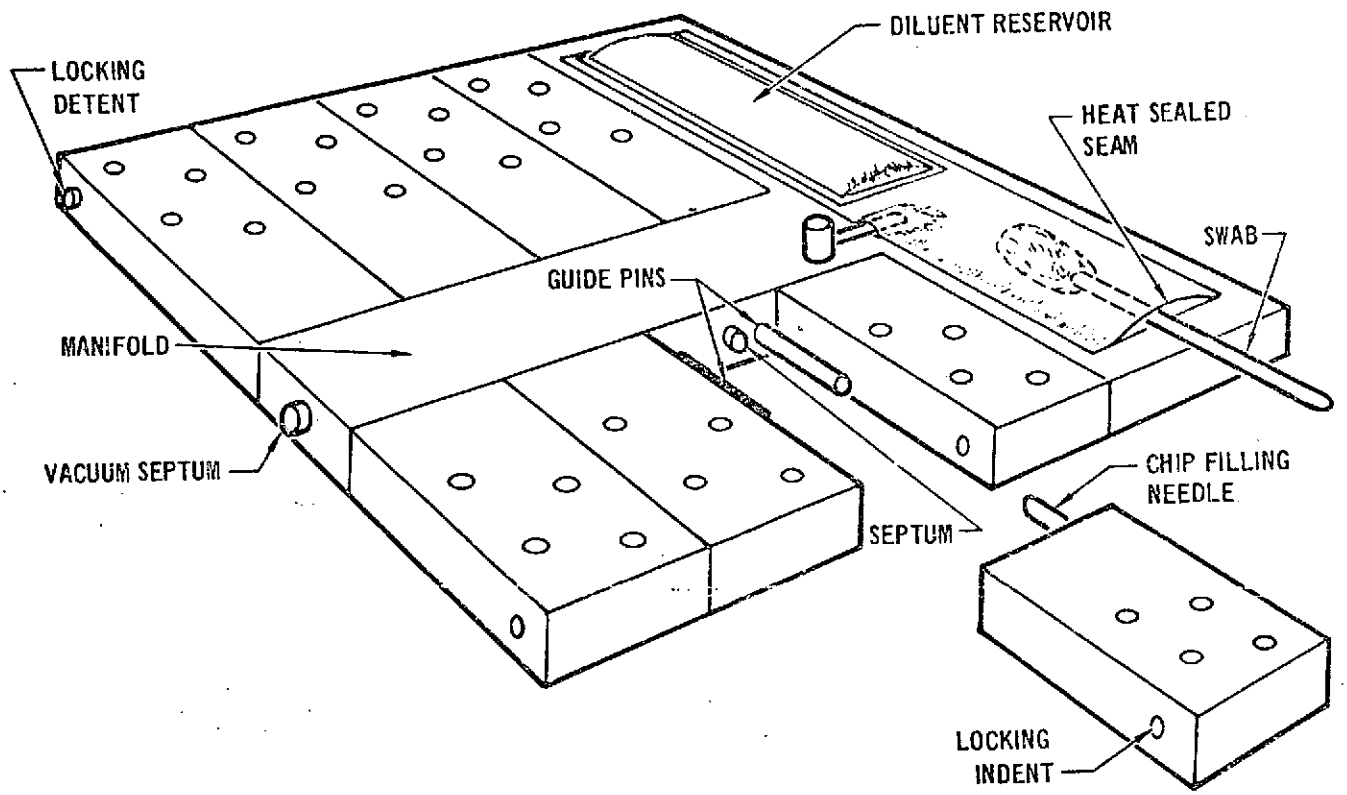
Concomitant microbiological studies performed with these devices indicate that filter passage simultaneously produces organism's migration, appropriate dilutions, and sample clarification.

Reproducibility is surprisingly good, though some filter paper types are demonstrated superior. We tested whatman numbers 1, 3, 42, and 541. We also tested Reeve Angel number 802, 934 AH (glass filter), and acetate membranes. Our subsequent testing, detailed under 3.3.7 Methods Development for Determining Selective Microorganism Numbers, proved asbestos fibers superior to all.

All devices discussed are designed to be conjunctively used with a diluent reservoir adjoining the filter device. Figure 22 shows the reservoir, consisting of an inner and outer bag. The outer bag encloses a sealed inner bag containing diluent. The outer bag is manifolded, to several filter chips. Growth test preparation are: inserting the filter device containing freese-dried nutrient into the connecting manifold; a swab holding the sample is placed in the outer reservoir bag, and its handle broken off and removed. The outer bag is then heat sealed; and the bag and chip evacuated via septum at the manifold end. After evacuation, the vacuum septum needle is removed. A clamp is applied to the bag-to-manifold tube; the inner diluent bag is broken, and the swab tip mixes the diluent. Following mixing, the clamp is removed, allowing sample-diluent mixture flow into the chips. Subsequently the dry nutrient is dissolved and the detection ports filled.

The described sampling devices are compact packages providing a selective media growth capability for variably concentrated microorganisms. We obtain different concentrations by diluent-sample mixture transport through filter material lengths. The result is a point referenced concentrarion gradient over distance. We simultaneously observe multiple detection port optical changes. These changes rapidly provide information regarding microorganisms species and original sample population numbers. The monitoring electro-optical instrumentation is capable of accommodating the detection ports' number dictated by nutrient and filter material properties. The instrumentation data output is in usable form and exactly time indexed. Multichannel optical system development paralleled multichannel device work. Subsequent paragraphs discuss this electronic development.

FIGURE 22
ADVANCED DESIGN OF RIGID MULTI-CHANNEL SAMPLE HOLDER
FOR SPACECRAFT USE



Many developmental changes evolved the filter device. Two major stages were: a) molded design, and b) machined design. In our first constructed device, several channels consisting of five filter strips separated and providing four detection ports sealed between two mylar polyethylene laminate layers. We then potted the laminate in polyester resin, obtaining a rigid package. Although preliminary testing demonstrated a well sealed device having good fluid transfer properties, it was difficult to accurately locate the filter strips and control detection port volume.

Overcoming these disadvantages, we mold blank polyester resin chips. We placed filter strips in chip slots and covered them with narrow Teflon adhesive tape strips, (Figure 23). We applied a second resin layer providing a rigid reliable seal. However we had difficulty controlling package thickness and fabrication time was excessive.

Figure 24 shows the machined device. It is machined from Lexan Polycarbonate (high density polyethylene could be used) and made reusable. The filter material is packed into cylindrical holes perpendicular to the detection port axis. Detection port depth is the machined chip thickness. We chose this device as the best MLM study design.

3.4.2.3 Rigid Sample Holder Development and Test - We call the rigid sample holders Filter Cassettes because of their complex function and design. These cassettes clarify and filter solids and semi-solids, serially dilute bacterial or fungal populations, and provide pre-programmed growth detection ports for selected bacteria or fungi.

Our fully developed cassette biological tests are presented with the (See: 3.5.3, MLM Sampling Devices Test and Evaluation) clinical specimen tests. Engineering evaluation is discussed in the following paragraphs.

We fabricated two sample systems including sample cassettes, diluent reservoir, sample mixing bag, and diluent distribution manifold for evaluating sample diluent mixing and filling techniques. The manifolds are designed to accept two 4-stage cassettes which may be monitored, two stages at a time, on the existing 6 channel laboratory model MLM. One of the designs has been filled reliably. A number of small bubbles are sometimes left in the cuvettes or may appear at short time after filling.

FIGURE 23
PHOTOGRAPH OF SEVEN CHANNEL RIGID SAMPLE
HOLDER MOLDED IN POLYESTER RESIN

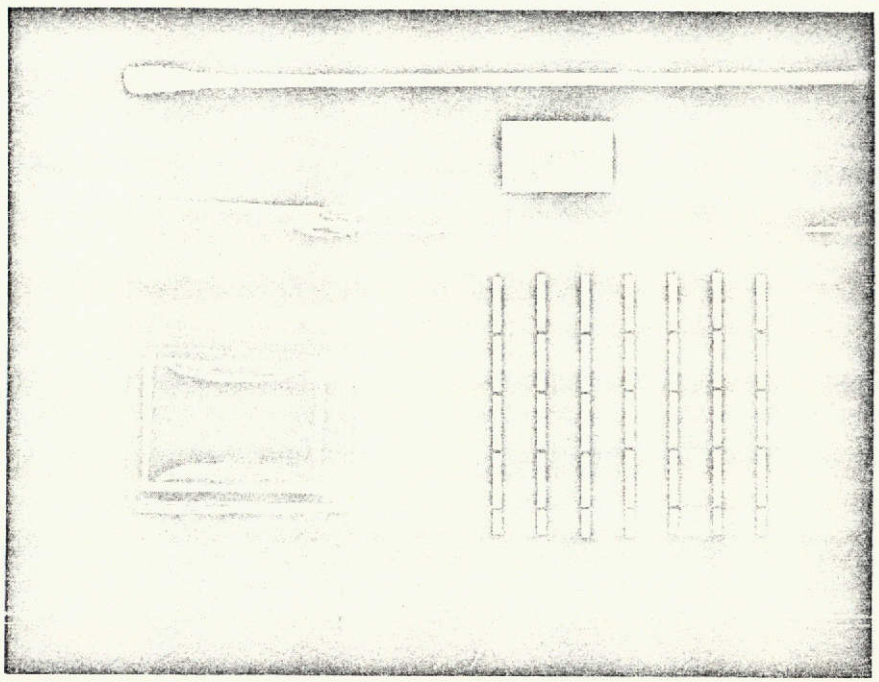
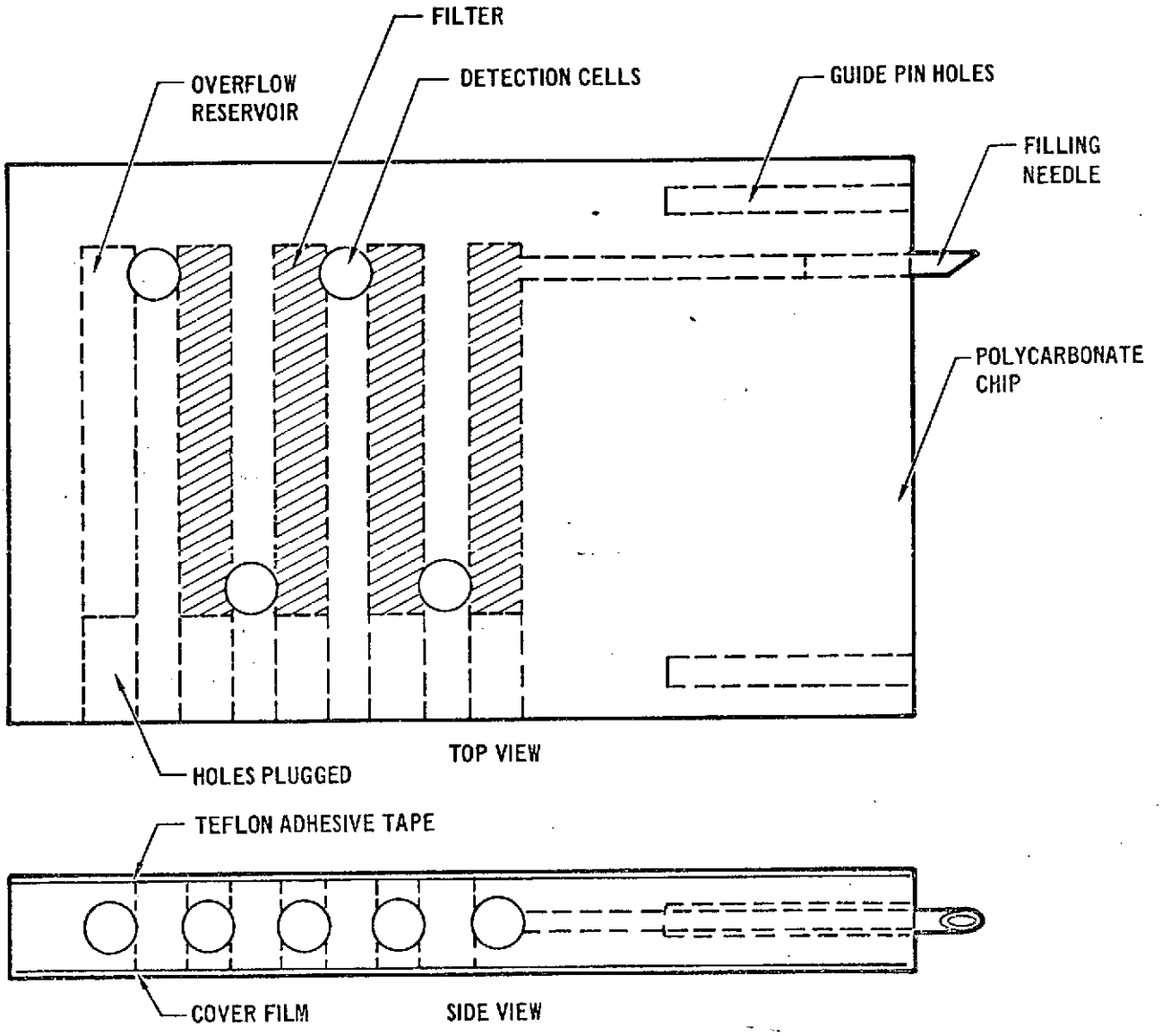


FIGURE 24
TOP AND SIDE VIEWS OF FILTER CHIP WITH FOUR DETECTION CELLS
THIS UNIT IS USED IN LABORATORY TESTS IN MLM



We avoid air bubbles presence in the detection cells wherever and whenever possible. Since bubbles have a migratory tendency, change shape, and coalesce in the detection cell, they tend to change light quantity (apparent density of the media) detector seen, which tends to give false readings.

Sources of bubbles are: 1) inadvertant air injection into the cassettes, 2) air remaining in the cassette while filling, 3) leaks, and 4) gas evolution during organism growth. Controlling evolved gas is presently beyond the cassette design realm. We eliminate moisture and air leaks through the materials and design choices. Inadvertent air injection into the cassette can be eliminated through good handling and filling techniques. The elimination of bubbles due to air in the cassette before filling is our most difficult bubble problem.

We can use four techniques, either singularly or together, to reduce the amount of air remaining in the cassette during filling: 1) the fluid pathway shape ,2) using a wetting agent on the pathway walls, 3) slow filling, and 4) cassette evacuation prior to filling.

The fluid pathway shape should place the detection cell exit opposite the entrance and should avoid sharp edges throughout. We are searching to find a microbes' and media compatible wetting agent. Slow filling will avoid violent mixing which can draw air from unfilled areas into the fluid. Cassette evacuation complicates the loading process and makes slow filling difficult, though tests have shown it can be done successfully.

3.4.2.4 Design and Test Required MLM Minor Modifications Improving Usefulness in Microorganism Identification - Multiple detection ports require a multi-channel instrument occupying minimal space and little operator attention. Our previously described models contained a separate, identical electronic channel for each detection port, requiring three calibration adjustments as a minimum. Consequently, extending this design to multiple channels required excessive space and operator time. Overcoming these disadvantages, we designed a sequential sampling MLM with only one electronic detector channel, requiring one adjustment and serving four detection ports. The major differences between this model and those developed previously are as follows:

- The detection ports are sampled sequentially rather than continuously.

- The calibration procedure is greatly simplified.
- The output data are digital displays.

We employed the basic electro-optical detection methodology present in earlier models. The sequential sampling model (Figure 25) amplifies and detects each detection port output signal. Our system samples each port for two minutes, every eight minutes, and the units' front panel displays the data. A one minute sample duration lower limit is dictated by electronic detection circuitry response, allowing switching transient decay before recording data.

After this first minute, the counters are reset (15 sec.) and then during the last forty five seconds of each two minute sample time, the data representing the optical density of the port being sampled is transferred and displayed on the front panel in the form of three decimal digits. The output display then blanks, at which time sampling of another detection port begins. In addition to the front panel temporary display (Figure 26), the data can be permanently recorded on an eight column auxiliary digital printer. Manual channel selection in lieu of automatic sampling is provided by switches on the front panel.

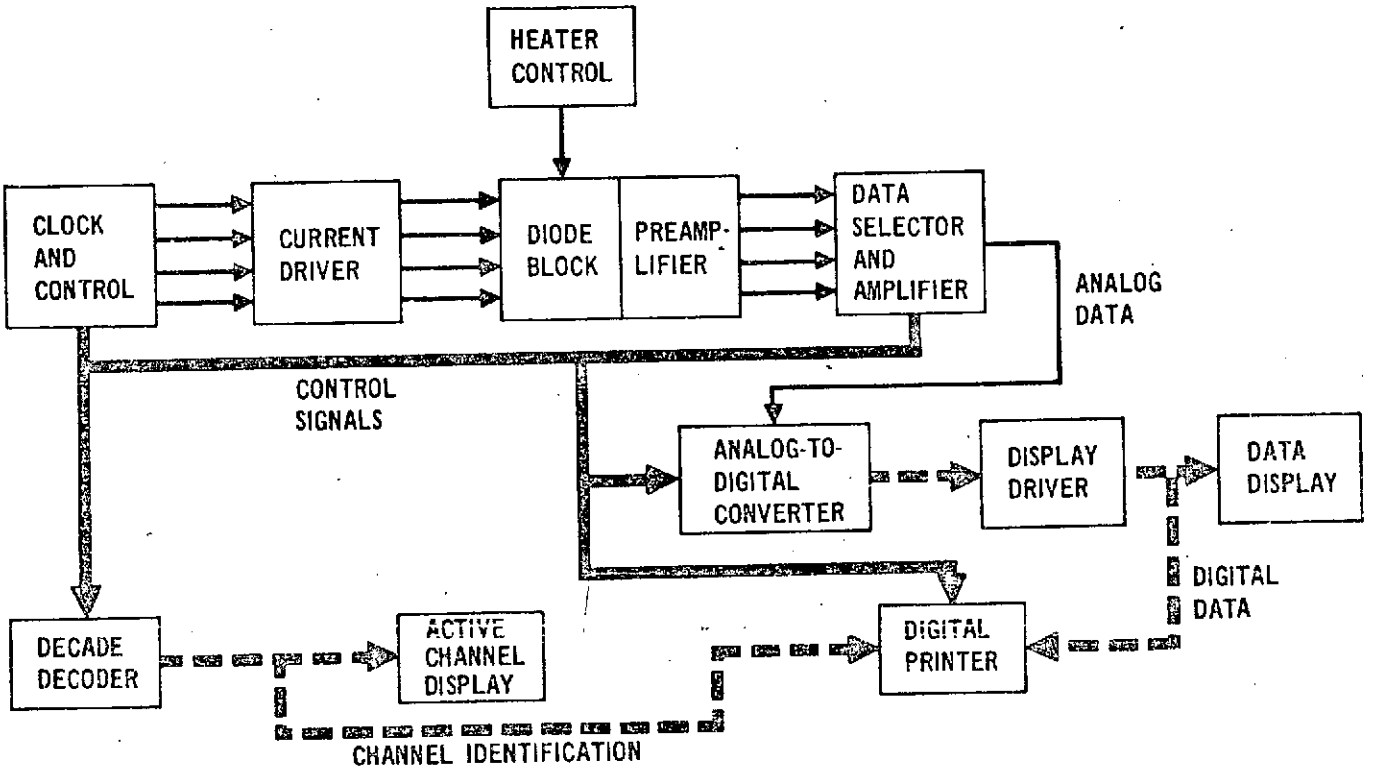
The analog-to-digital data conversion scale factors have been adjusted to accommodate the nutrient varieties used so far, and conform to previous models' accuracy. We currently regard this accuracy figure as one percent, established by the electronic detection circuitry stability. The sequential sampling model calibration procedure consists of throwing one switch and adjusting one control unit zeroing the output display. We facilitate this highly simplified procedure by employing a digital output display, yielding an expanded output scale.

The sequential sampling MLM consists of seven standard 3 x 3 inch circuit boards and a 6 x 9 inch control and display panel, contained in a 7 x 7 x 14 inch enclosure. Figure 27 shows the individual breadboard circuit cards described below.

Clock and Control - This circuit consists of digital integrated circuit packages forming clocks and counters which generate timing and control signals. The signal functions are to sequentially select the proper cell and to control the A/D converter and digital printer.

FIGURE 25

FOUR CHANNEL SEQUENTIAL SAMPLING MICROBIAL LOAD MONITOR
BLOCK DIAGRAM



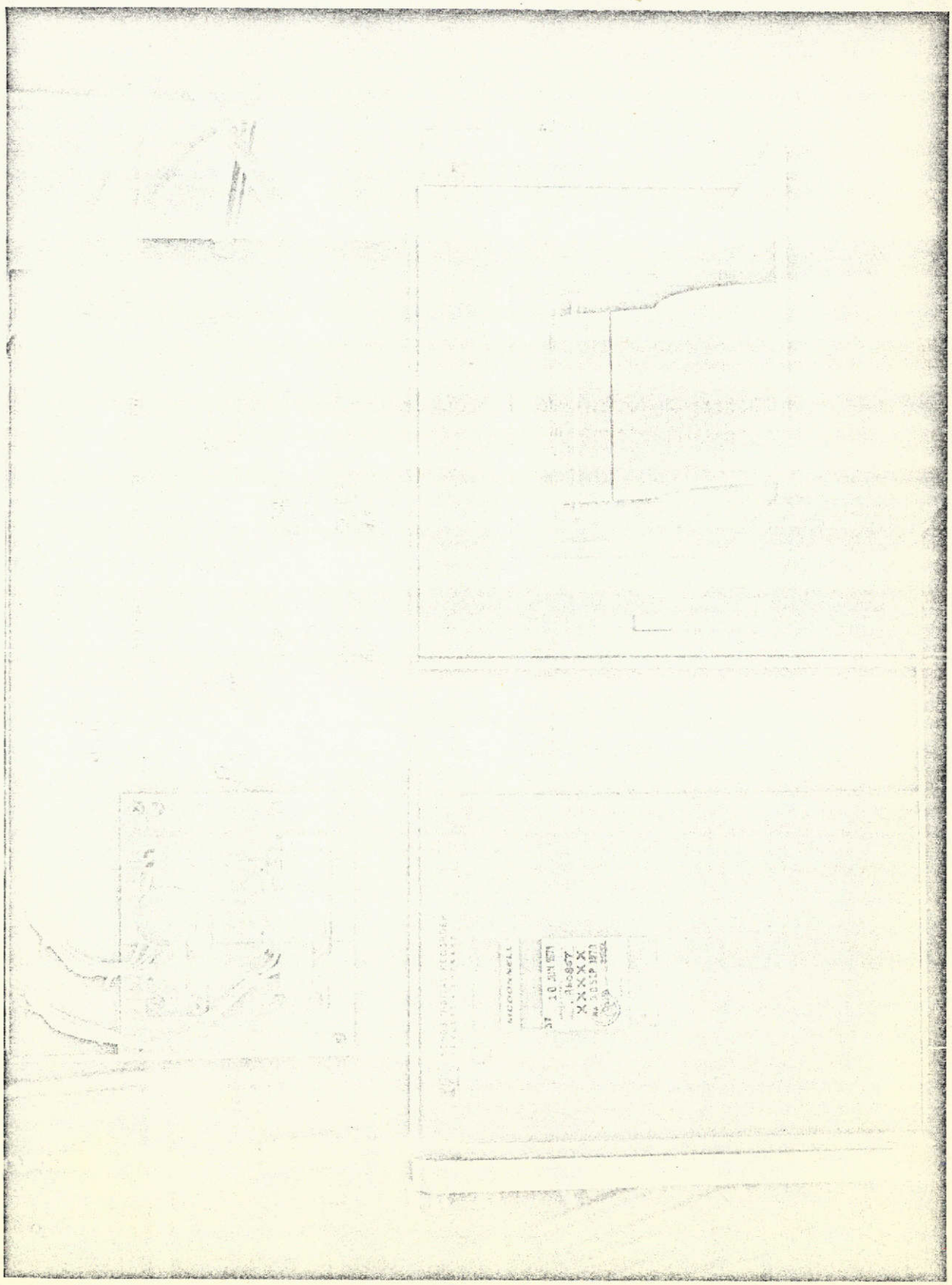
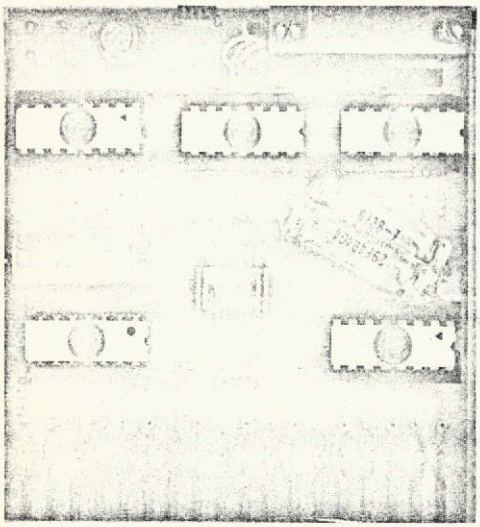
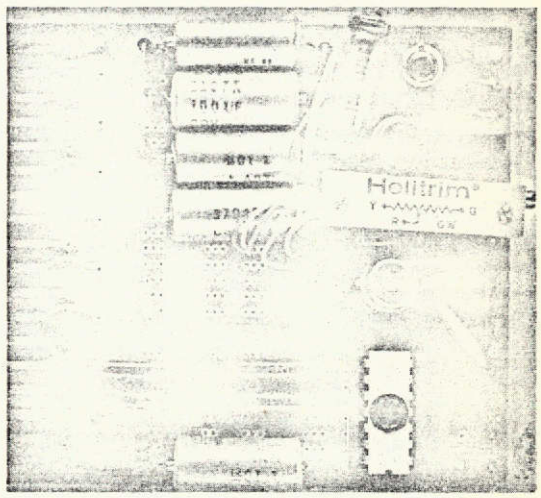


FIGURE 26
MLM FOUR CHANNEL SEQUENTIAL MONITOR AND PRINTOUT UNIT

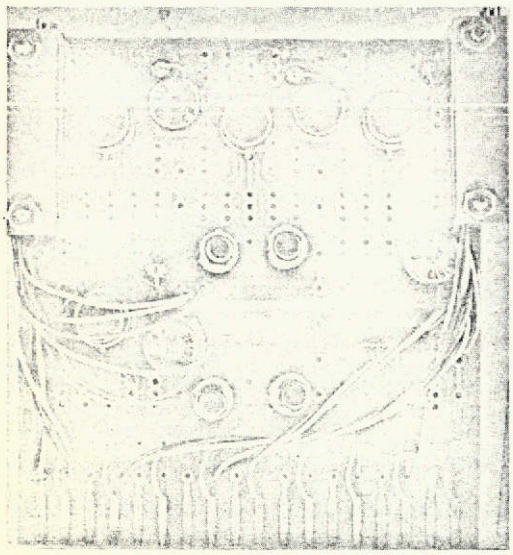
FIGURE 27
COMPLETED CIRCUIT BOARDS, SEQUENTIAL SAMPLING MLM



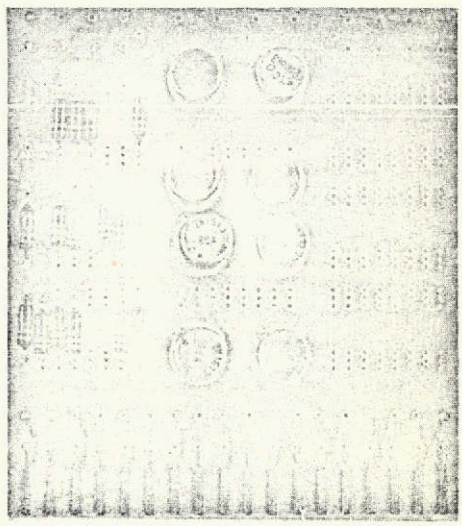
A. CLOCK AND CONTROL



B. DATA SELECTOR AND AMPLIFIER

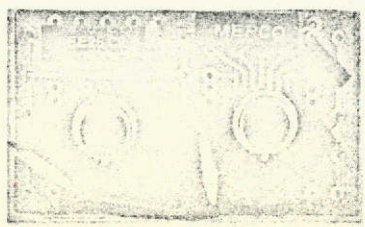


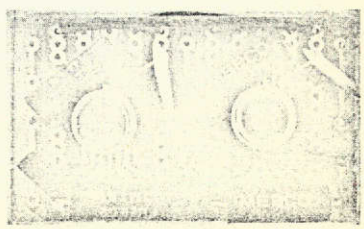
C. CURRENT DRIVER



D. DECADE DECODER

E. PREAMPLIFIERS





Level Converter - This circuit takes +12V logic voltages and converts them to +5V levels with sufficient current driving a digital printer. This conversion is used for all logic signals interfacing with the printer, (i.e. data, ch. I.D., and print control signal).

Elapsed Time Clock - These two boards generate elapsed time by counting down a clock and displaying the count on the front panel and the digital printer.

Current Driver - We modified the light emitting diodes' current driver circuit driving to accommodate an input clock signal change from bipolar to unipolar.

Decade Decoder - This circuit contains a 7 x 4 diode decoding matrix and eight transistors. Functionally it decodes a counter decimal output and controls the active cell number display.

Heater Control - These circuits accomplish detector block temperature control. The design is the same as the original MLM except all the heaters are on the negative voltage supply. This distributes the load currents between positive and negative supplies. (Indicators draw heavily on the positive supply.)

Data Selector and Amplifier - This circuit selects, amplifies, and detects a preamplifier output signal. The amplifier and detector portion is the same as the original MLM design.

Analog to Digital Converter - The A/D converter employes the ramp conversion technique changing analog output to a 12 bit BCD format. Each channel output is sequentially presented as a three-digit display.

Display Driver - This board's circuitry converts A/D converter logic signals to a level compatible with available BCD-to-7-segment decoders on the same board. The decoders furnish the proper drive currents to the solid state displays.

Control and Display Panel - This panel contains decimal indicators; and the necessary auto/manual selection and initial calibration switches and controls.

Detection Blocks and Pre-Amps - The detection blocks holding the photodiodes and sample cells are identical to the previous design. Preamplifiers

were redesigned, providing increased stability and fitting the same assembly. The improved preamplifiers have also been retrofitted into the existing laboratory model MLM units.

3.4.2.5 Breadboard System Test Results - We undertook several engineering tests evaluating the Sequential Sampling MLM (SSAMM) concept performance and acceptability. We performed these evaluation tests with the four breadboard channel SSAMM and they provide the basic design input data for an expanded laboratory model development. Specifically, our test program included: (1) determining the short term electronic stability requirement for stable A/D conversion, (2) evaluating the effect of sampling rate and resulting transients on sample period length, (3) system performance degradation resulting from the light emitting diodes, wide electrical and mechanical tolerances, and (4) analyzing changes in system performance caused by modifications providing parts count and power requirement reduction. Our test results provide the basis for several design improvements described below.

We made one calibration procedure change. Instead of balancing the amplifiers for zero digital output reading (000), we found a reading of at least 001 is necessary. This results from an ambiguity which allows a negative voltage to also give a zero reading. In addition, we installed a switch enabling the operator to have ten to one cycle speedup, reducing checkout and setup time requirements.

a) Stability Test Results - Our initial SSAMM design used four separate clocks. One for elapsed time, a second for a channel and A/D control signals, a third for modulation and demodulation, and a fourth for A/D conversion counting. With this arrangement stability tests showed that one conversion to the next could differ by as much as 10 counts (over 1%). We discovered this was due to millivolts of 10 KHz analog signal ripple. We discarded the separate A/D clock in favor of the 10KHz modulator clock. We subsequently found the count difference to be about ± 1 count or 0.1%.

b) Sample Rate Test Results - A filter circuit analysis indicates that in 4 or 5 seconds, the analog voltage should be within 0.01% of its final value. Stability tests indicate that leaving a channel in manual mode continues to decrease analog voltage for at least 30 seconds. The reason

seems to result from individual emitter diode warm up times upon application of the 10 KHz drive current (i.e. thermal stabilization with its inherent effect on light output). This would have little effect on synchronous operation since the same time point would be sampled. If the cycle times were changed, its value would be different (for example 980 with a 7.5 second stabilization time before conversion compared to 996 with a 75 second stabilization time). We found the 75 second time to be within ±1 count of the value at 5 minutes, when placed in the manual mode.

c) Light Emitting Diode (LED) Electrical and Mechanical Tolerances - The four channel SSAMM was examined for detected output versus LED drive current. The results indicate that individual diodes may require as much as 3 to 1 difference in drive current for equal preamplifier output with no intervening sample. Selection of 4 LEDs from a group of ten reduced the drive current range required from 6 to 1 to the present level of 3 to 1. The epoxy lens quality control seems to be the chief varient source. Misalignment of the blocks as presently configured could cause a 11 or 10% difference in count in initial setup, but thereafter would remain constant.

d) Results of Modifications - We reduced parts count by eliminating the separate A/D clock. Similar results may be achieved by combining the elapsed time and A/D control clock. We redesigned the preamplifier circuit, lowering the detector diode impedance and giving the capability of some fixed gain. The detected waveshape is greatly improved by latter change and the LED current driver replacement with a higher slew rate operational amplifier.

3.5 MLM Experimental Studies

Demonstrating the MLM diagnostic capability using actual clinical samples, we designed the following studies. For this capability, the developed MLM selective media will detect the appropriate organisms seeded into actual clinical specimens. We also demonstrated that media selectively includes naturally occurring organisms as well as laboratory strains. Our success is detailed below.

3.5.1 Evaluating Clinical Sample Identification Procedures - We tested culture media developed for detecting specific organisms with pure cultures, mixed cultures, and human throat, urine, and fecal clinical samples.

We scheduled candidate culture media testing at the fifth contract month end. We began these tests on schedule. Figure 28 presents a methods and techniques flow diagram used in our study. Our scheme permits determining number and kinds of medically important organisms present in the throat swabs; and provides throat culture seeding with known organism numbers.

Table 10 presents the MLM results and standard procedure tests for determining medically important organisms' presence in four throat cultures each from four different subjects. Organisms present giving a positive MLM culture media reaction were undetected by standard screening tests.

Seeding known microorganism numbers into the throat swab inoculum was our next procedure. We performed microtest plate tests and MLM tests. Table 11 presents our results. In throat culture number one, Staphylococcus aureus and Klebsiella Pneumoniae were added to the inoculum.

The MLM media we tested were Mannitol Salt, Urease, Cetrimide, Coliform, Mycosel, and Synthetic for Fungi. S. aureus should give a positive reaction only in Mannitol Salt. K.pneumonaie should give a positive reaction in urease medium but not a typical Proteus type reaction; good growth should occur in coliform medium and a strong precipitate typical of coliform organisms. We anticipated the results exactly, with broth subcultures revealing correct detection and identification.

In throat culture number two, we seeded the inoculum with Candida albicans and Pseudomonas aeruginosa. We predicted our results, except a slight Urease medium turbidity occurred, subsequently identified as P. aeruginosa.

Our tests were successful, however, the slight P. aeruginosa growth in the wrong (Urease) medium may have been due to transport medium buffers, and the inoculum volume/growth medium volume ratio being too high. In other words, the Urease medium selective agent concentrations may have been diluted by adding too much inoculum. In subsequent tests we accounted for these results.

In the second test series we tried seeded specimens with the completely developed MLM systsms. That is, we pre-programmed a growth and detection cassette freeze-dried medium; a clinical specimen was seeded with known organism amounts and then we used MLM to detect organism kinds and numbers present. Figures 29 and 30 present Urine study data.

FIGURE 28

FLOW DIAGRAM FOR TESTING THROAT CULTURES IN MLM

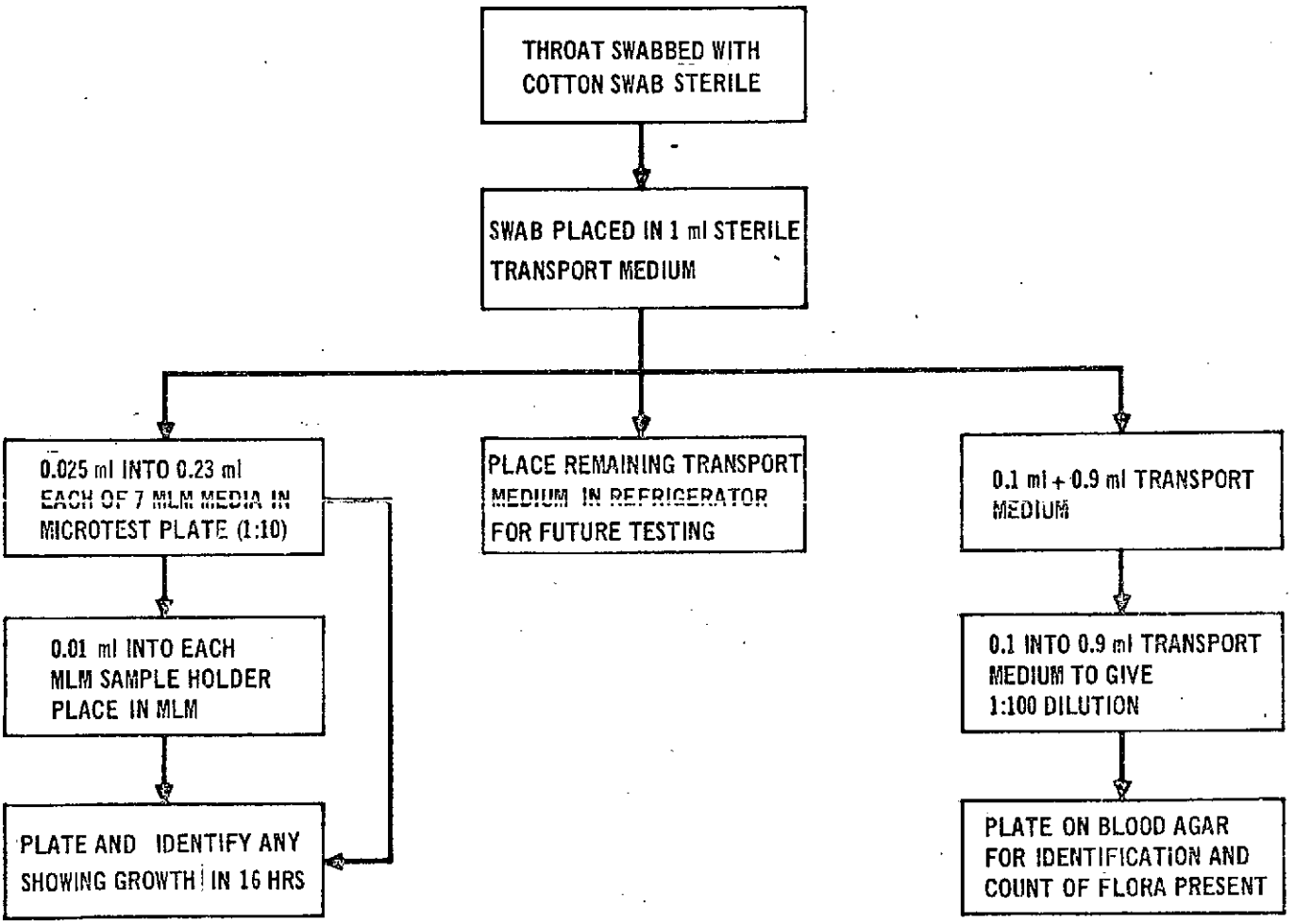


TABLE 10
RESULTS OF THROAT CULTURE SCREENING

THROAT CULTURE NO.	MANNITOL SALTS		MITIS SALIVARIUS		CETRIMIDE		UREASE		COLIFORM		MYCOSEL		SYNTHETIC FOR	FUNGI	STANDARD CULTURE BLOOD AGAR
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	
1	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	5×10^5/ml α STREPTOCOCCUS 5×10^4/ml NEISSERIA SP 1×10^4/ml GAFFKYA TETRAGENA 1×10^4/ml γ STREPTOCOCCUS
2	NG	X	NG	X	NG	X	NG	X	NG	X	NG	X	NG	X	2×10^3/ml α STREPTOCOCCUS 1×10^3/ml DIPHTHEROIDS
3	NG	X	NG	X	NG	X	NG	X	NG	X	NG	X	NG	X	1×10^5/ml α STREPTOCOCCUS 1×10^5/ml DIPLOCOCCUS PNEUMONIAE 1×10^2/ml DIPLOBACILLUS (g+)
4	NG	X	NG	X	NG	X	NG	X	NG	X	NG	X	NG	X	1×10^5/ml α STREPTOCOCCUS 2×10^4/ml NEISSERIA SP

A = MICROTEST PLATE
B = MLM

NG = NO GROWTH
X = NOT DONE

TABLE 1

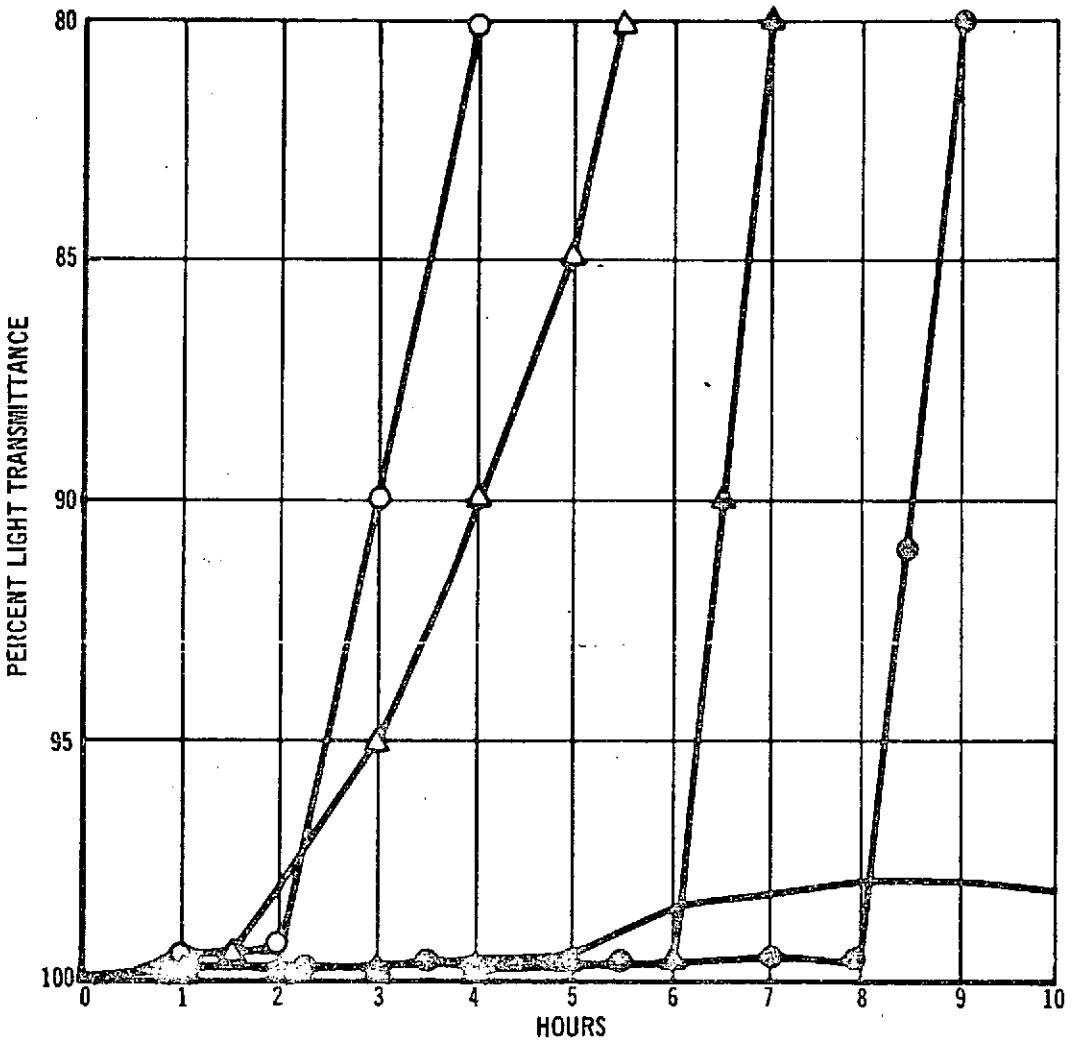
RESULTS OF SEEDING KNOWN ORGANISMS INTO THROAT CULTURES FOR MLM DETECTION STUDY

THROAT CULTURE NUMBER AND ORGANISMS SEEDED	MLM DETECTION * RESULTS IN CULTURE MEDIA LISTED BELOW	SUBCULTURE RESULTS
	MANNITOL SALT UREASE CETRIMIDE COLIFORM MYCOSEL SYNTHETIC FOR FUNGI	
THROAT CULTURE NO. 1 SEEDED { 1×10^7/ml STAPHYLOCOCCUS AUREUS; 1×10^6/ml KLEBSIELLA PNEUMONIAE	+ + − + − − (11 HR) (13 HR) (10.5 HR)	STAPHYLOCOCCUS AUREUS RECOVERED FROM MANNITOL SALT MEDIUM. KLEBSIELLA PNEUMONIAE RECOVERED FROM UREASE AND COLIFORM MEDIUM
THROAT CULTURE NO. 2 SEEDED { 1×10^6/ml CANDIDA ALBICANS; 1×10^7/ml PSEUDOMONAS AERUGINOSA	− ± + ± + ± (16 HR) (13 HR) NOTE: ± MEANS SLIGHT GROWTH DETECTED BUT NOT TYPICAL FOR POSITIVE TEST	CANDIDA ALBICANS WAS RECOVERED FROM MYCOSEL AND SYNTHETIC MEDIUM. PSEUDOMONAS WAS RECOVERED FROM CETRIMIDE, UREASE AND COLIFORM BROTH

*ALL TESTS ALSO PERFORMED IN MICROTEST PLATES AS WELL AS MLM. RESULTS OF SUBCULTURE AND DETECTION PARALLEL RESULTS IN MLM

FIGURE 29

SEQUENTIAL DETECTION BY MLM OF ESCHERICHIA COLI GROWING IN FILTRATION CASSETTES



CONTROL; COLIFORM MEDIUM

GROWTH I CHAMBER — COLIFORM MEDIUM + APPROX. 10^6 E. COLI ORG/ML

GROWTH II CHAMBER — COLIFORM MEDIUM + APPROX 10^5 E. COLI ORG/ML

GROWTH III CHAMBER — COLIFORM MEDIUM + APPROX 10^4 E. COLI ORG/ML

GROWTH IV CHAMBER — COLIFORM MEDIUM + APPROX 10^3 E. COLI ORG/ML

FIGURE 30
MLM DETECTION OF ESCHERICHIA COLI IN HUMAN URINE

——CONTROL; FREEZE DRIED COLIFORM MEDIUM REHYDRATED WITH 1/10 DILUTION OF URINE AND DISTILLED WATER; PH 6.8

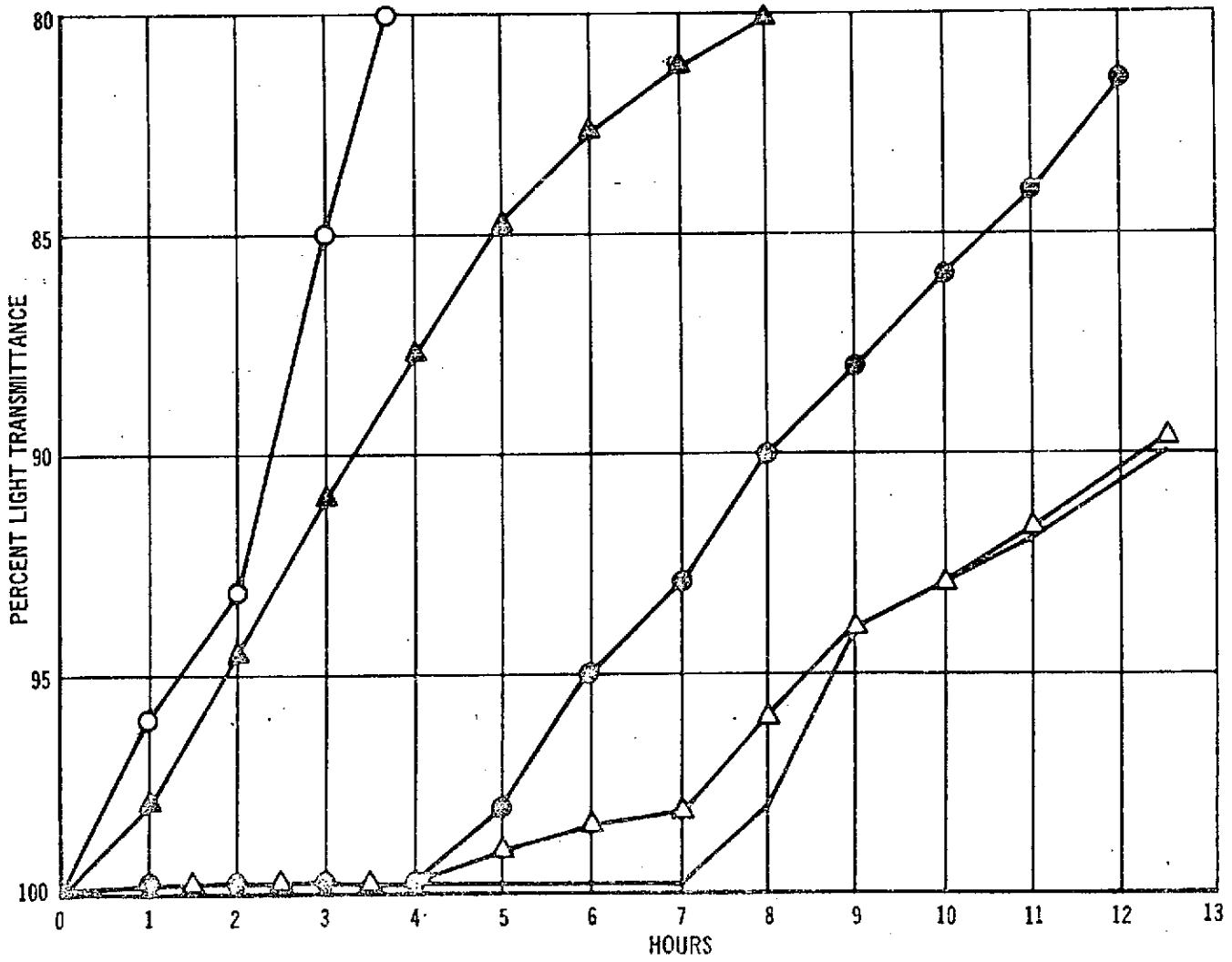
O—O GROWTH CHAMBER I; REHYDRATED MEDIUM SEEDED WITH 10^3 E. COLI ORG/ML

△—△ GROWTH CHAMBER II; REHYDRATED MEDIUM SEEDED WITH 10^2 E. COLI ORG/ML

⊕—⊕ GROWTH CHAMBER III; REHYDRATED MEDIUM SEEDED WITH $< 10^1$ E. COLI ORG/ML

△—△ GROWTH CHAMBER IV; REHYDRATED MEDIUM SEEDED WITH 0 E. COLI ORG/ML

DETECTION AND GROWTH CASSETTES PRE-PROGRAMMED WITH DRIED MEDIUM. FILTRATION BEDS WERE PRESENT BETWEEN GROWTH CHAMBERS



We acquired feces data by diluting the feces 1:10 with water and rehydrating the cassette with this mixture. The cassette contained Salmonella, freeze-dried medium, and filtration beds. We divided the diluted samples into two portions. One portion was our control; we seeded the other portion with known S. paratyphi B. amounts.

As a result of several MLM Salmonella tests, we chose Salmonella paratyphi B. Figure 31 presents these data. Our results demonstrate paratyphi B as the choice salmonella for rapid detection.

Figure 32 presents our fecal sample seeding results. The only chamber yielding a positive reaction was the detection chamber containing approximately 10^2 Salmonella paratyphi B. The curve differs from the study results presented in Figure 31. However, we used different organism concentrations - our fecal study utilizing approximately 10^3 less organisms.

3.5.2 Evaluate Procedures Using Naturally Occurring Organisms - After completing the preliminary tests, we decided that MLM selective media development had advanced sufficiently to justify large scale testing.

We made arrangements with a local hospital to obtain clinical specimens; recent pathogenic organism isolates; and access to hospital analyzed clinical specimen records.

For the study, we placed one tenth ml media aliquots in microtest plate wells (total volume 0.3 ml). The media were freeze-dried. After freeze-drying, we covered the microtest plates with adhesive plastic film and stored them at 4°C. We used the following freeze-dried media: Coliform, Cetrimide, Salmonella, Urease, Staphylococcus, Salicin-Neomycin, Mycosel, and Synthetic for Fungi.

We did not freeze-dry Herellea broth, but added it to microtest plates later.

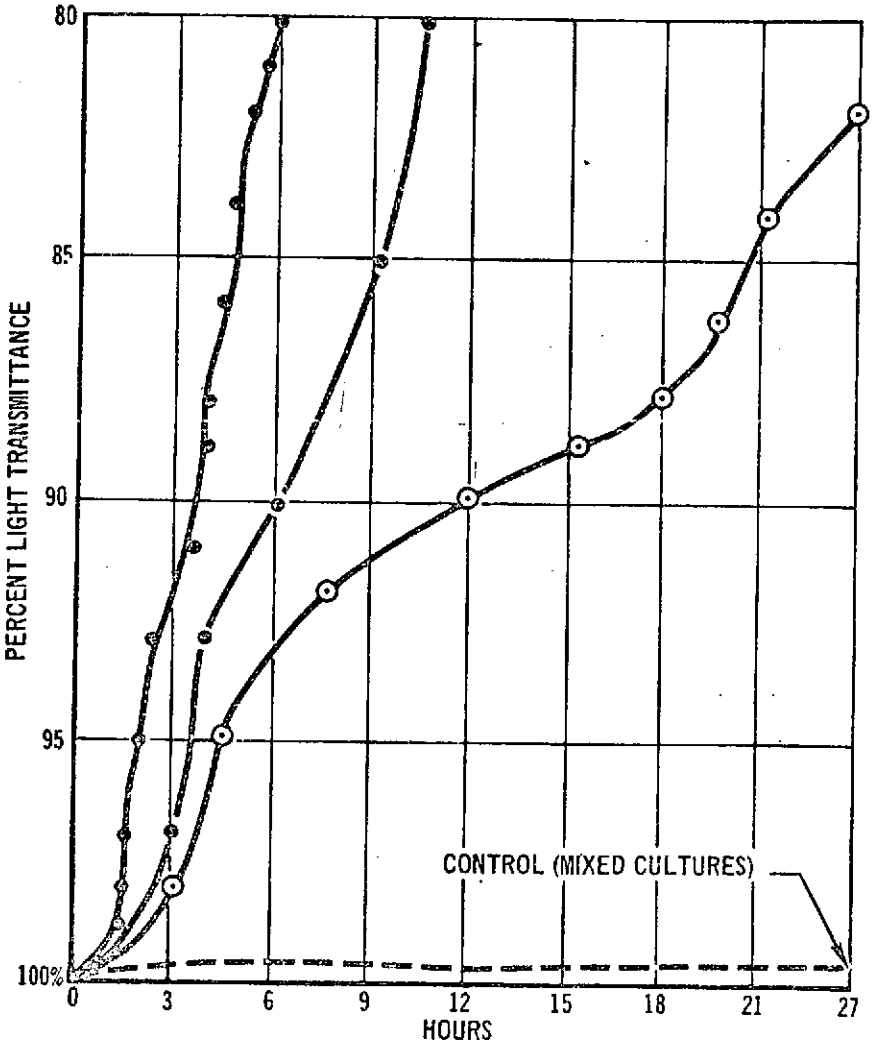
We prepared clinical specimens obtained from hospital patients as follows:

Urine	Dilution 1:10 in sterile neutral distilled water.
Sputum	Swab dipped in specimen; mixed in 1 ml sterile neutral distilled water.
Throat	Swab mixed in 1 ml sterile neutral distilled water.

FIGURE 31
SALMONELLA MLM CULTURE MEDIUM TESTS WITH MIXED CULTURES AND VARIOUS SALMONELLA SPECIES

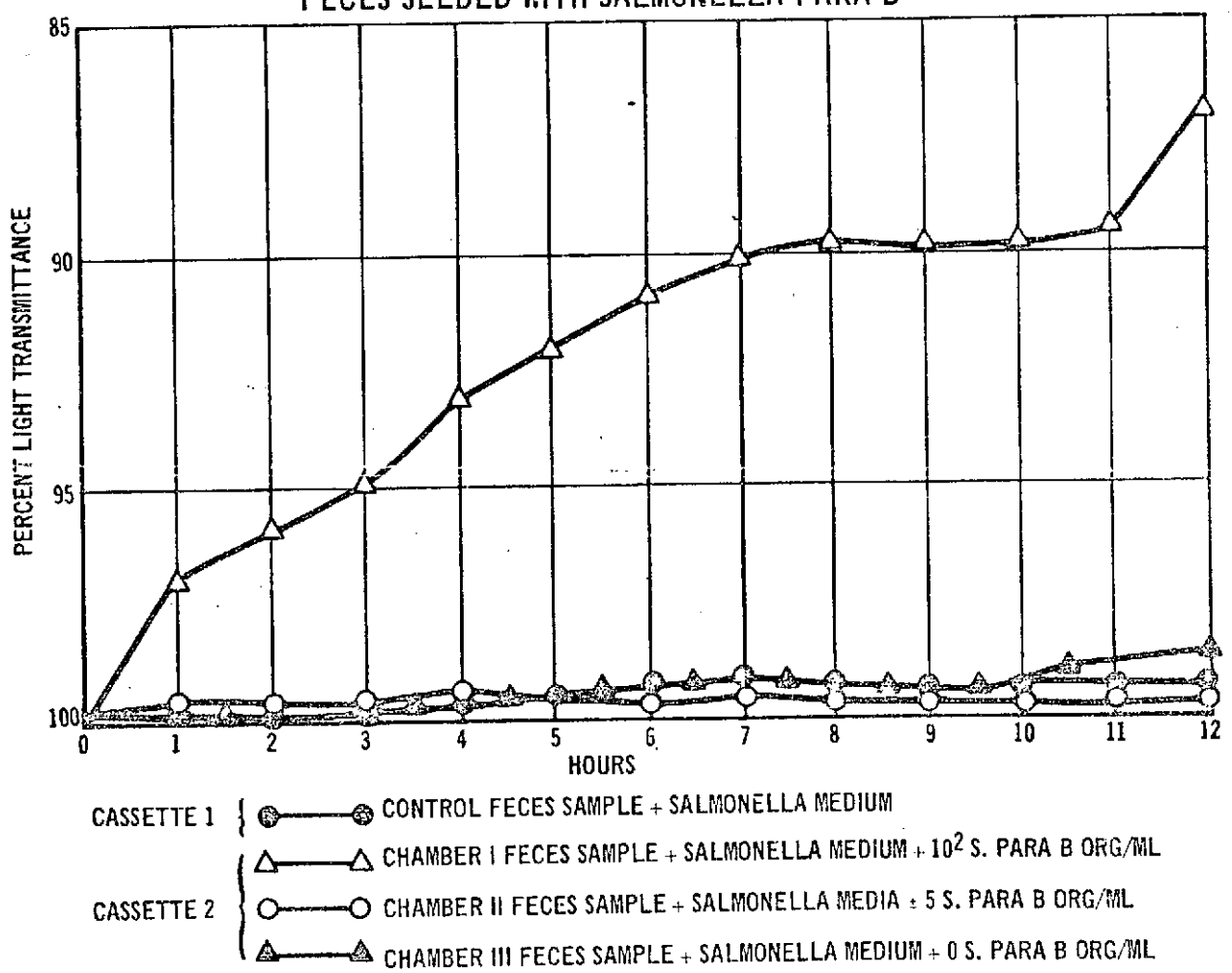
MIXED CULTURE ORGANISMS:

- CITROBACTER SP.
- ENTEROBACTER SP.
- PROTEUS SP.
- ESCHERICHIA COLI
- PSEUDOMONAS AERUGINOSA
- PROVIDENCIA SP.
- AEROBACTER SP.
- SERRATIA MARCESCENS
- FLAVOBACTERIUM SP.
- SHIGELLA SP.



- CONTROL CONTAINING ONLY MIXED CULTURES
- SALMONELLA PARA B + MIXED CULTURES
- SALMONELLA TYPHOSA + MIXED CULTURES
- SALMONELLA SCHOTTMUELLERI + MIXED CULTURES

FIGURE 32
MLM DETECTION OF SALMONELLA IN HUMAN
FECES SEEDED WITH SALMONELLA PARA B



Wound — Swab mixed in 1 ml sterile neutral distilled water.

Feces — Measured amount* in 3 ml sterile neutral distilled water.

Filtration to remove any large particles.

* A fairly simple fecal sample "measuring spoon" yielded a standard test aliquot. We devised a microcapillary plunger tube, permitting approximately equal-sized aliquot acquisition.

We added one tenth ml of the water mixed specimen to each of the eight freeze-dried wells. Three hundredths ml was added to 0.27 ml Herellea broth. We incubated inoculated broths 16 hours at 37°C. For identification, we streaked all positive cultures on MacConkey Agar and Blood Phenylethyl Agar.

A local hospital microbiology lab performed the following standard tests on the same specimens:

Throat swab Sputum samples	MacConkey agar Sheep Blood agar Chocolate agar Thioglycollate broth	CO_2 Environment
Wound swab	MacConkey agar Blood agar - anaerobic culture Blood phenylethyl agar Thioglycollate broth	
Urine Sample - 0.01 ml plated on:	Blood agar MacConkey agar Thioglycollate broth	
Fecal sample	Blood phenylethyl agar SS agar MacConkey agar H&E agar Selenite broth 18 hrs.	

We performed MLM media testing as follows:

MICROTEST CULTURE PROCEDURE

0.1 ml uring	Measured fecal	Sputum swab,
0.9 ml Distilled H_2O	aliquot mixed in	throat swab or
	3 ml Distilled H_2O	wound swab mixed
		in 1 ml distilled H_2O

Coarst filtration, removing large particles

0.1 ml added to each freeze-dried media in microtest wells.

Incubate 37°C

At 16 hrs. - Plate all positives for identification comparison with standard culture results.

Results are presented in Tables 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24.

TABLE 12

MLM CULTURE MEDIA STUDIES WITH HUMAN THROAT SWABS

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMO NELLA	BETA STREPTOCOCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
1.	THROAT	0	0	0	0	0	0	0	0	0	1. MODERATE GROWTH E.COLI 2. MOD.GROWTH NORMAL FLORA*
2.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	MODERATE GROWTH NORMAL FLORA
3.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	MODERATE GROWTH NORMAL FLORA
4.	THROAT	0	0	0	0	0	0	0	0	0	MODERATE GROWTH NORMAL FLORA
5.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	MODERATE GROWTH NORMAL FLORA
6.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	± CANDIDA ALBICANS	+ CANDIDA ALBICANS	0	1. MODERATE GROWTH YEAST IDENTIFIED AS CANDIDA ALBICANS 2. NORMAL FLORA
7.	THROAT	± KLEBSIELLA PNEUMONIAE	+ PSEUDOMONAS AERUGINOSA**	+ PROTEUS SP.	0	0	0	0	0	0	1. LIGHT GROWTH KLEBSIELLA PNEUMONIAE 2. LIGHT GROWTH PROTEUS SP. 3. MODERATE GROWTH ALPHA STREPTOCOCCI 4. LIGHT GROWTH NEISSERIA CATARRHALIS

* THOSE ORGANISMS INCLUDED IN "NORMAL FLORA" ARE:
GAMMA STREP, α STREP, NEISSERIA, DIPHTHEROIDS, AND GAFFKYA TETRAGENA.

** CETRIMIDE BROTH REVEALED PSEUDOMONAS NOT FOUND IN STANDARD CULTURE

TABLE 13
MLM CULTURE MEDIA STUDIES WITH HUMAN THROAT SWABS (Continued)

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMO-NELLA	BETA STREPTOCOCCUS	STAPHYLOCOCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
8.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
9.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
10.	THROAT	0	0	0	0	0	0	+ CANDIDA ALBICANS	± CANDIDA ALBICANS	0	NORMAL FLORA
11.	THROAT	0	0	0	0	0	+ STAPHYLOCOCCUS AUREUS		0	0	1. LIGHT GROWTH S.AUREUS, COAGULASE POSITIVE 2. MODERATE GROWTH NORMAL FLORA
12.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCI	0	0	0	0	NORMAL FLORA
13.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
14.	THROAT	+ E.COLI	0	0	0	0	0	0	0	0	1. MODERATE GROWTH E.COLI 2. NORMAL FLORA
15.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCI FEW BETA STREPTOCOCCI	0	0	0	0	1. MODERATE GROWTH BETA STREPTOCOCCUS GROUP A 2. NORMAL FLORA
16.	THROAT	0	0	0	0	0	0	0	0	0	1. MODERATE GROWTH DIPLOCOCCUS PNEUMONIAE

* MLM MYCOSEL MEDIUM REVEALED CANDIDA ALBICANS NOT DETECTED IN ROUTINE CULTURE.

TABLE 14
MLM CULTURE MEDIA STUDIES WITH HUMAN THROAT SWABS (Continued)

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMO-NELLA	BETA STREPTOCOCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
17.	THROAT	0	0	0	0	0	0		0	0	NORMAL FLORA
18.	THROAT	+KLEBSIELLA PNEUMONIAE	0	±KLEBSIELLA PNEUMONIAE	0	0	0		0	0	1. MODERATE GROWTH KLEBSIELLA PNEUMONIAE 2. HEAVY GROWTH DIPLOCOCCUS PNEUMONIAE 3. HEAVY GROWTH YEAST NOT CANDIDA ALBICANS
19.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
20.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
21.	THROAT	+ E. COLI KLEBSIELLA PNEUMONIAE	0	± KLEBSIELLA PNEUMONIAE	0	0	0	0	0	0	1. MODERATE GROWTH KLEBSIELLA PNEUMONIAE 2. LIGHT GROWTH E. COLI
22.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	NORMAL FLORA
23.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
24.	THROAT	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	1. HEAVY GROWTH HAEMOPHILUS INFLUENZAE 2. MODERATE GROWTH NORMAL FLORA
25.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
26.	THROAT	0	0	0	0	0	0	0	0	0	NORMAL FLORA
27.	THROAT	0	0	0	0	0	0	0	0	0	HEAVY GROWTH DIPLOCOCCUS PNEUMONIAE LIGHT GROWTH NORMAL FLORA

TABLE 15

MLM CULTURE MEDIA STUDIES WITH HUMAN URINE

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTOCOCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
1.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
2.	URINE	0	0	+ PROTEUS SP.	0	+γSTREP.	0	0	0	0	75,000/ML* PROTEUS
3.	URINE	+ E.COLI	0	0	0	0	0	0	0	0	10,000/ML E. COLI
4.	URINE	+ E. COLI	0	0	0	0	0	0	0	0	75,000/ML E.COLI
5.	URINE	0	+ PSEUDOMONAS AERUGINOSA	± PROTEUS SP.	0	0	0	0	0	0	1. 50,000/ML PSEUDOMONAS AERUGINOSA 2. 50,000/ML PROTEUS SP.
6.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
7.	URINE	+ENTEROBACTER SP.	+ FEW PROTEUS SP.	+ ENTEROBACTER SP. PROTEUS SP.	0	- STREP. FAECALIS	0	0	0	0	1. 25,000/ML ENTERBACTER SP. 2. 10,000/ML PROTEUS SP.
8.	URINE	0	0	0	0	- STREP. FAECALIS	0	0	0	0	75,000/ML STREPTOCOCCUS FAECALIS
9.	URINE	0	0	0	0	0	0	0	0	0	<100/ML STAPHYLOCOCCUS ALBUS
10.	URINE	+ E.COLI	0	0	0	0	0	0	0	0	90,000/ML E. COLI
11.	URINE	0	0	0	0	- STREP. FAECALIS	0	0	0	0	1. 1500/ML STREPTOCOCCUS FAECALIS 2. 5000/ML ALPHA STREPTOCOCCI

* MLM MEDIA RESULTS ON A 1:10 DILUTION OF SPECIMEN. THEREFORE, STANDARD CULTURE OF 75,000/ML OR 7.5×10^4/ML IN MLM MEDIA WOULD BE 7.5×10^3/ML

TABLE 16
MLM CULTURE MEDIA STUDIES WITH HUMAN URINE (Continued)

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	SALICIN	STAPHYLO-COCCUS	MYCOSEL	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
12.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
13.	URINE	0	0	0	0	0	0	0	0	0	100/ML E. COLI
14.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
15.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
16.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
17.	URINE	+ E.COLI	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
18.	URINE	+ E.COLI	+PSEUDOMONAS AERUGINOSA	0	0	0	0	0	0	0	1. 25,000/ML E. COLI 2. 10,000/ML PROVIDENCE SP
19.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
20.	URINE	+ E. COLI	0	0	0	0	0	0	0	0	1. 50,000/ML PROTEUS* SP 2. 10,000/ML E. COLI
21.	URINE	+ENTEROBACTER SP.	0	0	0	0	0	0	0	0	1. 10,000/ML ENTEROBACTER SP. 2. 5,000/ML PSEUDOMONAS AERUGINOSA**
22.	URINE	+ E.COLI	0	0	0	0	0	0	0	0	600/ML E. COLI
23.	URINE	0	0	0	0	0	0	0	0	0	900/ML STAPHYLOCOCCUS ALBUS COAGULASE NEGATIVE
24.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS

*5000/ML (5×10^3/ML) PROTEUS NOT DETECTED IN UREASE BROTH
**500/ML PSEUDOMONAS NOT DETECTED IN MLM BROTH.

TABLE 17
MLM CULTURE MEDIA STUDIES WITH HUMAN URINE (Continued)

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTO-COCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
25.	URINE	+E.COLI	+E. COLI*	+ FEW −E.COLI	0	0	0	0	0	0	100,000/ML E. COLI
26.	URINE	0	0	0	0	0	0	0	0	0	200/ ML GAMMA STREPTOCOCCI
27.	URINE	0	0	0	0	0	0	0	0	0	700/ML STAPHYLOCOCCUS ALBUS COAGULASE NEGATIVE
28.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
29.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
30.	URINE	0	0	0	0	0	+ FEW −S. ALBUS	0	0	0	NO GROWTH 24 HOURS
31.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
32.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
33.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
34.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
35.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
36.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS

*500/ML C. ALBICANS NOT DETECTED IN MYCOSEL BROTH

TABLE 18
MLM CULTURE MEDIA STUDIES WITH HUMAN URINE (Continued)

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTO-COCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
37.	URINE	0	0	0	0	0	0	0	0	0	1. 5000 ML STREP 2. 2000 ML STAPHYLOCOCCUS ALBUS, COAGULASE NEGATIVE
38.	URINE	+E.COLI	0	0	0	0	0	0	0	0	80,000 ML E. COLI
39.	URINE	+ E.COLI	0	0	0	0	0	0	0	0	100,000 ML E. COLI
40.	URINE	+ENTEROBACTER SP.	0	± ENTEROBACTER SP	0	0	0	0	0	0	90,000 ML ENTEROBACTER SP.
41.	URINE	+ CITROBACTER SP.	0	± RARE ENTEROBACTER SP.	± CITRO-BACTER SP.	0	0	0	0	0	75,000 ML CITROBACTER SP. 1000 ML ENTEROBACTER SP.
42.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
43.	URINE	+ E. COLI	0	0	0	0	0	0	0	0	100,000/ML E. COLI
44.	URINE	+ENTEROBACTER SP.	0	± ENTEROBACTER SP.	0	0	0	0	0	0	100,000/ML ENTEROBACTER
45.	URINE	+E. COLI	0	0	0	0	0	0	0	0	80,000/ML E. COLI
46.	URINE	+ E.COLI	0	0	0	0	0	0	0	0	50,0000 ML E. COLI
47.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
48.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS

TABLE 19
MLM CULTURE MEDIA STUDIES WITH HUMAN URINE (Continued)

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTO-COCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
49.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
50.	URINE	0	0	+ PROTEUS SP.	0	+ BETA STREPTO-COCCI	0	0	0	0	1. 25,000/ML PROTEUS SP. 2. 25,000/ML BETA STREPTO-COCCI, NOT GROUP A
51.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
52.	URINE	+ E. COLI	0	0	0	0	0	0	0	0	100,000/ML E. COLI
53.	URINE	+ E. COLI	0	± PROTEUS SP.	0	0	0	0	0	0	1. 75,000/ML E. COLI 2. 50,000/ML PROTEUS
54.	URINE	0	0	0*	0	0	0	0	0	0	5,000/ ML PROTEUS SP.
55.	URINE	+ E. COLI	0	+ PROTEUS SP.**	0	0	0	0	0	0	1. 50,000/ML E. COLI 2. 10,000/ML ENTERBACTER SP. 3. 5,000/ML PROTEUS SP.
56.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
57.	URINE	+ E.COLI	0	± E. COLI	0	0	0	0.	0	0	1. 25,000/ML E. COLI 2. 7,500/ML STAPHYLOCOCCUS ALBUS, COAGULASE NEGATIVE

* 500/ML PROTEUS NOT DETECTED IN MLM BROTH
** 500/ML PROTEUS DETECTED IN MLM BROTH

TABLE 20
MLM CULTURE MEDIA STUDIES WITH HUMAN URINE (Continued)

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTO-COCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
58.	URINE	0	0	0	0	0	0	0*	0	0	5,000/ML CANDIDA ALBICANS *
59.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
60.	URINE	+ENTEROBACTER SP.	0	±ENTEROBACTER SP.	0	0	0	0	0	0	100,000/ML ENTEROBACTER SP.
61.	URINE	0	+ PSEUDO-MONAS AERUGINOSA	+ PROTEUS SP.	0	0	0	0	0	0	1. 50,000/ML PSEUDOMONAS AERUGINOSA 2. 100,000/ML PROTEUS SP.
62.	URINE	+ENTEROBACTER SP.	0	+PROTEUS SP. ENTEROBACTER SP.	0	0	0	0	0	0	1. 900/ML ENTEROBACTER SP. 2. 2500/ML PROTEUS SP.
63.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
64.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
65.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS
66.	URINE	+ ENTEROBACTER SP.	0	±ENTEROBACTER SP.	0	0	0	0	0	0	75,000/ML ENTEROBACTER SP.
67.	URINE	+ E. COLI	0	0	0	0	0	0	0	0	100,000/ML E COLI
68.	URINE	+ E. COLI	0	+ PROTEUS SP. E. COLI	0	0	0	0	0	0	1. 75,000/ML E. COLI 2. 30,000/ML PROTEUS SP.
69.	URINE	0	+PSEUDO MONAS	+ FEW PROTEUS SP.	0	0	0	0	0	0	100,000/ML PSEUDOMONAS AERUGINOSA.
70.	URINE	+ E. COLI	0	± E.COLI	0	0	0	0	0	0	60,000/ML E. COLI
71.	URINE	0	0	0	0	0	0	0	0	0	NO GROWTH 24 HOURS

*500/ML C. ALBICANS NOT DETECTED IN CANDIDA BROTH

TABLE 21

MLM CULTURE MEDIA STUDIES WITH WOUNDS FROM HUMAN SOURCES

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTO-COCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
1	WOUND	+ENTEROBACTER SP.	+PSEUDOMONAS AERUGINOSA	+ENTEROBACTER SP.	0	0	0	0	0	0	1. MODERATE GROWTH PSEUDOMONAS AERUGINOSA 2. LIGHT GROWTH ENTEROBACTER SP.
2	WOUND	E. COLI +ENTEROBACTER SP.	0	+ENTEROBACTER SP.*	0	+ BETA -STREPTO-COCCI	+ S. ALBUS BETA STREPTO-COCCI	0	0	0	1. MODERATE GROWTH E. COLI 2. LIGHT GROWTH PROTEUS SP. 3. LIGHT GROWTH ENTEROBACTER SP. 4. LIGHT GROWTH BETA STREPTOCOCCI 5. LIGHT GROWTH STAPHYLOCOCCUS ALBUS
3	WOUND	0	0	0	0	0	STAPHYLO-COCCUS AUREUS	0	0	0	MODERATE GROWTH STAPHYLO-COCCUS AUREUS COAGULASE POSITIVE
4	WOUND	0	0	+ PROTEUS SP.	0	0	0	0	0	0	MODERATE GROWTH PROTEUS SP.
5	WOUND	0	0	0	0	0	0	0	0	0	VERY LIGHT GROWTH ENTEROBACTER SP.
6	EAR	0	+ PSEUDOMONAS AERUGINOSA	0*	0	0	+STAPHYLO-COCCUS AUREUS	0	0	0	1. MODERATE GROWTH PSEUDOMONAS 2. LIGHT GROWTH PROTEUS SP.

*LIGHT GROWTH PROTEUS (STANDARD CULTURE RESULTS) NOT DETECTED IN MLM BROTH.

TABLE 22

MLM CULTURE MEDIA STUDIES WITH WOUNDS FROM HUMAN SOURCES

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTO-COCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
7	WOUND	+ E. COLI	0	0	0	0	+ FEW E. COLI	0	0	0	HEAVY GROWTH E. COLI
8	WOUND	0	0	0	0	0	0	0	0	0	MODERATE GROWTH GAMMA STREPTOCOCCI
9	WOUND	0	0	0	0	0	+ STAPHYLO-COCCUS AUREUS	0	0	0	MODERATE GROWTH STAPHYLO-COCCUS AUREUS COAGULASE POSITIVE
10	EYE SWAB	0	0	0	0	+ FEW STAPHYLO-COCCUS AUREUS	+ STAPHYLO-COCCUS AUREUS	0	0	0	MODERATE GROWTH STAPHYLO-COCCUS AUREUS, COAGULASE POSITIVE
11	WOUND	0	0	0	0	0	0	0	0	0	GRAM POSITIVE ANAEROBIC, RODS NOT YET IDENTIFIED
12	WOUND	0	0	0	0	+ STREPTO-COCCUS FAECALIS	0	0	0	0	HEAVY GROWTH STREPTOCOCCUS FAECALIS
13	WOUND	0	0	0	0	0	0	0	0	0	LIGHT GROWTH BACTEROIDES SP.

TABLE 23
MLM CULTURE MEDIA STUDIES WITH HUMAN SPUTUM

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTO-COCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
1.	SPUTUM	KLEBSIELLA PNEUMONIAE	+PSEUDOMONAS AERUGINOSA	+PROTEUS SP.	0	0	0	0	0	0	1. MODERATE GROWTH KLEBSIELLA PNEUMONIAE 2. LIGHT GROWTH PROTEUS SP.
2.	SPUTUM	0	0	0	0	0	+STAPHYLO- * COCCUS AUREUS	0	0	0	NORMAL FLORA
3.	SPUTUM	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	1. HEAVY GROWTH HAEMOPHILUS INFLUENZAE 2. NORMAL FLORA
4.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
5.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
6.	SPUTUM	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	NORMAL FLORA
7.	SPUTUM	0	0	0	0	0	0	0	0	0	1. LIGHT GROWTH DIPLOCOCCUS PNEUMONIAE 2. NORMAL FLORA
8.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
9.	SPUTUM	+K. PNEUMONIAE	+P. AERUGINOSA **	+K. PNEUMONIAE	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	1. LIGHT GROWTH KLEBSIELLA PNEUMONIAE 2. NORMAL FLORA
10.	SPUTUM	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	NORMAL FLORA
11.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
12.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
13.	SPUTUM	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	HEAVY GROWTH HAEMOPHILUS INFLUENZAE
14.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
15.	SPUTUM	+ K. PNEUMONIAE	0	0	0	0	0	0	0	0	LIGHT GROWTH KLEBSIELLA PNEUMONIAE
16.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
17.	SPUTUM	0	0	0	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	MODERATE GROWTH DIPLOCOCCUS PNEUMONIAE
18.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
19.	SPUTUM	+ K. PNEUMONIAE	+ P. AERUGINOSA	+ K. PNEUMONIAE	0	+ GAMMA STREPTOCOCCUS	0	0	0	0	1. HEAVY GROWTH KLEBSIELLA PNEUMONIAE 2. HEAVY GROWTH PSEUDOMONAS AERUGINOSA
20.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA
21.	SPUTUM	0	+ P. AERUGINOSA	P. AERUGINOSA	0	0	0	0	0	0	HEAVY GROWTH P. AERUGINOSA
22.	SPUTUM	0	0	0	0	0	0	0	0	0	NORMAL FLORA

*STAPHYLOCOCCUS MEDIUM REVEALED S. AEREUS NOT DETECTED IN STANDARD CULTURE
** CETRIMIDE BROTH REVEALED PSEUDOMONAS NOT DETECTED IN STANDARD CULTURE

TABLE 24
MLM CULTURE MEDIA STUDIES WITH HUMAN FECES

NO.	SPECIMEN	COLIFORM	CETRIMIDE	UREASE	SALMONELLA	BETA STREPTOCOCCUS	STAPHYLO-COCCUS	CANDIDA	SYNTHETIC FUNGI	HERELLEA	STANDARD CULTURE RESULTS
1.	FECES	+ ENTEROBACTER SP.	0	± ENTEROBACTER SP.	0	0	0	0	0	0	NO ENTERIC PATHOGENS
2.	FECES	ENTEROBACTER SP. + E. COLI	+ PSEUDOMONAS AERUGINOSA	+ ENTEROBACTER SP.	0	+ GAMMA STREPTO-COCCUS	0	0	0	0	NO ENTERIC PATHOGENS
3.	FECES (SWAB)	0	0	0	0	0	+ S. AUREUS*	0	0	0	NO ENTERIC PATHOGENS
4	FECES	+ E. COLI	0	0	0	+ S. FAECALIS	0	0	0	0	SALMONELLA GROUP B (TYPHIMURIUM)
5.	FECES	ENTEROBACTER SP. + E. COLI	0	± ENTEROBACTER SP.	0	0	0	0	0	0	NO ENTERIC PATHOGENS
6.	FECES	+ E. COLI	0	0	0	0	0	0	0	0	NO ENTERIC PATHOGENS
7.	FECES	ENTEROBACTER SP. + E. COLI	0	ENTEROBACTER SP. + PROTEUS	0	0	0	0	0	0	NO ENTERIC PATHOGENS
8.	FECES	+ ENTEROBACTER SP.	0	0	0	+ S. FAECALIS	0	0	0	0	NO ENTERIC PATHOGENS
9.	FECES	E. COLI + ENTEROBACTER SP.	0	± ENTEROBACTER SP.	0	0	0	0	0	0	NO ENTERIC PATHOGENS

*STAPHYLOCOCCUS MEDIUM REVEALED S. AUREUS NOT ISOLATED IN STANDARD CULTURE

Data inspection reveals the MLM culture media to have excellent selectivity in spite of the clinical material presence. In certain cases the MLM media detected organisms not detected by standard techniques. Examples are the following:

1. Throat culture - C. albicans in Mycosel.
2. Throat culture - Pseudomonas in Cetrimide.
3. Fecal culture - S. aureaus in Mannitol Salts.
4. Sputum - S. aureus in Mannitol Salts.
5. Sputum - Pseudomonas in Cetrimide.
6. Ear swab - S. aureus in Mannitol Salts.

At least 3 of the above cannot be attributed to contamination, as both isolates of Pseudomonas came from patients whose previous routine cultures contained Pseudomonas.

Throat culture revealed 2 - 3 yeast colonies not reported in routine culture.

Possible contamination cannot be ruled out in the remaining three cultures revealing S. aureus, as no previous patient history was available. On the other hand, a few organisms were detected by the standard tests, but were missed by our MLM media. These are as follows:

1. Wound Swab - standard culture revealed light growth of Proteus not detected in Urease broth.
2. Ear swab - Same as 1 above.
3. Fecal sample - standard culture revealed S. typhimurium, a Salmonella species that will not grow in the MLM salmonella broth.

(In other cases we detected Proteus present in 1000/ml and 500/ml amounts.) The reason for the Nos. 1, 2, and 4 failures is unknown.

In another series of our tests, recent cultures (24-72 hrs.) were obtained from a local hospital. These organisms were isolated from throat, sputum, bronchial washings, urine, feces, and wounds. The organisms were subcultured and after incubating 16-24 hours, normal saline slurries are made giving

approximately 10^6 organisms/ml. Each organism was tested for growth or positive reaction with each of the following MLM media.

- Coliform Broth - We proved this medium 100 percent reliable. All E. coli, Enterobacter/Aerobacter, and Klebsiella cultures tested (total 84) were positive in the broth. The remaining 125 gram positive and gram negative organisms did not grow. One culture, E. coli mutabile, a late lactose fermenter, did not grow within 16 hours.
- Cetrimide Broth - This medium showed total selectivity for Pseudomonas aeruginosa. All 16 cultures of Pseudomonas grew. The remaining 185 gram positive and gram negative organisms did not grow within 16 hours.
- Staphylococcus Broth - All 14 Staphylococcus aureus cultures grew well. Nine of 15 S. epidermidis cultures showed light growth in 16 hours but no coagulum. However, three cultures of Streptococcus faecalis and one culture of P. aeruginosa also grew lightly.
- Salicin-Neomycin Broth - All 22 beta hemolytic streptococci, and gamma streptococci grew well. In two cases gram negative rods were isolated from the broth after 16 hours. (Enterobacter and Proteus).
- Salmonella Broth - One of the seven Salmonella cultures tested did not grow. However, it was already known that S. typhimurium was capable of only limited broth growth. This is because the culture medium was primarily designed to select S. typhosa and Paratyphi A and B.
- Herellea Broth - The single Herellea culture obtained during this study grew moderately well.
- Synthetic Fungi Broth - No recent Aspergillus niger cultures were available for this test. However, none of the 209 gram positive and gram negative organisms tested grew in the broth.

- Mycosel Broth - No recent yeast cultures were obtained for the test. Two Enterobacter/Aerobacter cultures, two P. aeruginosa cultures and one S. aureus culture grew. We modified the broth formula by adding 250 mcg/ml Colymycin and 250 mcg/ml Naladixic Acid (final concentrations). We made the same additions to the Synthetic broth as it was observed that some gram negative rods would grow in this broth after 16 hours.
- Proteus broth - The previously reported formula and two variations were used for this experiment, however the variations were unsatisfactory.

The above test results are summarized in Table 25. Table 26 presents further data analysis showing the fresh isolate media reliability. Special note should be made of the streptococci Salicin-Neomycin Broth. Conceptually, this medium's reliability depends upon adding two percent blood cells. This formulation should yield complete identification of beta hemolytic streptococci growing alone or in the presence of gamma streptococci. For the studies presented, no blood was added because the tests were performed at the hospital laboratory without growth curve recording instrumentation. Results are presented from observations after 16 hours incubation. It is apparent that the percent of false positives is higher than if blood had been added and growth curve record had been made. From such curves it would be possible to differentiate beta streptococci from all other organisms present. In spite of less than ideal conditions, MLM media false positives ranged from 3.3 to 0 percent, while known specific organisms' detection ranged from 86 to 100 percent.

3.5.3 Test and Evaluate the Performance of Sampling Devices with the MLM in Identification Studies - Our success in all previous testing, documented in foregoing sections, were combined into the Filter Detection cassette. A sampling device whose design provides automatic filtration, clarification, dilution, and growth and detection cells MLM compatable. These cassettes were preprogrammed with freeze-dried media, outfitted with asbestos filter beds, and were vacuum filled with inoculum from appropriate clinical specimens. A photograph of a sample cartridge is presented in Figure 33. The plastic diluent bag is connected to the vacuum manifold to which the filter cassettes are attached. After vacuum application, release of a valve (round dark spot

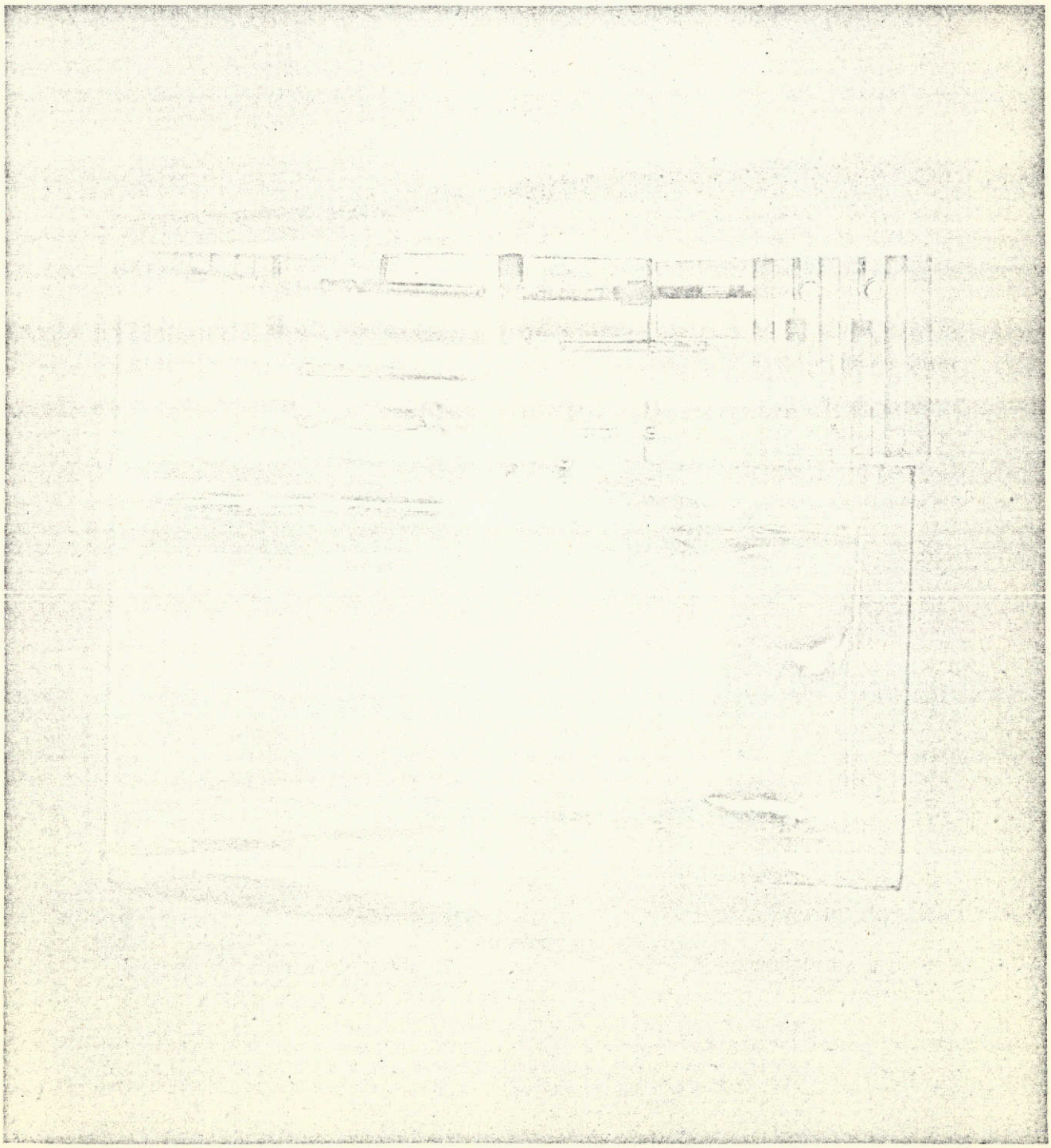
TABLE 25
RELIABILITY OF MLM CULTURE MEDIA WITH CLINICAL ISOLATES

MEDIUM	NUMBER OF ISOLATES TESTED FOR WHICH MEDIUM IS SPECIFIC	POSITIVES		NEGATIVES		FALSE POSITIVES (GROWTH OF OTHER ORGANISMS)	
		NO.	%	NO.	%	NUMBER	%
COLIFORM	84	84	100%	0	0%	0	0%
CETRIMIDE	16	16	100%	0	0%	0	0%
STAPHYLOCOCCUS	14	14	100%	0	0%	4 (4/209–14 x 100)	2.1%
BETA STREPTOCOCCUS	22	22	100%	0	0%	6 (6/209–22 x 100)	3.3%
SYNTHETIC–FUNGI	0	–	–	–	–	0	0%
SALMONELLA	7	6	86%	1	14%	0	0%
CANDIDA	0	–	–	–	–	5 (5/209 x 100)	2.4%
HERELLEA	1	1	100%	0	0%	0	0%

TABLE 26
SELECTIVITY OF MLM CULTURE MEDIA WITH FRESH CLINICAL ISOLATES

CLINICAL ISOLATE	NUMBER TESTED	COLIFORM POS	COLIFORM NEG	CETRIMIDE POS	CETRIMIDE NEG	STAPHYLO-COCCUS POS	STAPHYLO-COCCUS NEG	BETA STREPTO-COCCUS POS	BETA STREPTO-COCCUS NEG	SALMONELLA POS	SALMONELLA NEG	SYNTHETIC FUNGI POS	SYNTHETIC FUNGI NEG	CANDIDA POS	CANDIDA NEG	HERELLEA POS	HERELLEA NEG
ESCHERICHIA COLI	43	43	0	0	43	0	43	0	43	0	43	0	43	0	43	0	43
ENTEROBACTER/AEROBACTER SP.	39	39	0	0	39	0	39	1±	38	0	39	0	39	2	37	0	43
KLEBSIELLA PNEUMONIAE	2	2	0	0	2	0	2	0	2	0	2	0	2	0	2	0	2
PSEUDOMONAS AERUGINOSA	16	0	16	16	0	1	15	0	16	0	16	0	16	2	14	1±	15
PROTEUS SP.	14	0	14	0	14	0	14	1±	13	0	14	0	14	0	14	0	14
STAPHYLOCOCCUS AUREUS (COAGULASE +)	14	0	14	0	14	14	0	3±	11	0	14	0	14	1±	13	0	14
STAPHYLOCOCCUS EPIDERMIDIS (COAGULASE –)	15	0	15	0	15	9±	6	0	15	0	15	0	15	0	15	0	15
STREPTOCOCCUS FAECALIS	6	0	6	0	6	3±	3	6	0	0	6	0	6	0	6	0	6
GAMMA STREPTOCOCCUS	8	0	8	0	8	0	8	8	0	0	8	0	8	0	8	0	8
ALPHA STREPTOCOCCUS	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7
BETA STREPTOCOCCUS GRP.A	8	0	8	0	8	0	8	8	0	0	8	0	8	0	8	0	8
DIPLOCOCCUS PNEUMONIAE	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7
GAFFKYA TETRAGENA	4	0	4	0	4	0	4	1±	3	0	4	0	4	0	4	0	4
NEISSERIA GONORRHOEAE	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
NEISSERIA SP.	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7
HAEMOPHILUS INFLUENZAE	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
HAEMOPHILUS PARAINFLUENZAE	3	0	3	0	3	0	3	0	3	0	3	0	3	0	3	0	3
DIPHTHEROIDS	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5
STREPTOBACILLUS SP.	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
SALMONELLA SP.	7	0	7	0	7	0	7	0	7	6	1	0	7	0	7	0	7
HERELLEA	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	1	0
TOTAL	209																

FIGURE 33
MLM SAMPLE CARTRIDGE



near center) permits the diluted clinical sample to fill each filter cassette. As the fluid passes into the cassette, filter beds clarify and serially dilute the clinical inoculum. Freeze-dried media contained in the filtration beds dissolves and transforms the fluid into selective culture media. The cassettes are then MLM placed for organisms detection.

Filter cassettes in conjunction with the sample cartridge permit the MLM to become a diagnostic instrument. Medically important specific organisms can be detected and their relative numbers determined.

Documentation of results with clinical specimens seed with known organisms is as follows:

a) We seeded Staphylococcus aureus (coagulase positive) into human feces, Figure 34, human urine, Figure 35, and human throat swab inoculum, Figures 36, 37. S. aureus was detected in all three samples in a matter of hours. In all cases, system sensitivity seemed to be from 10 to 100 organisms.

b) We seeded Salmonella typhosa into human urine, Figure 38. As with S. aureus, sensitivity limit seemed to be about 100 organisms.

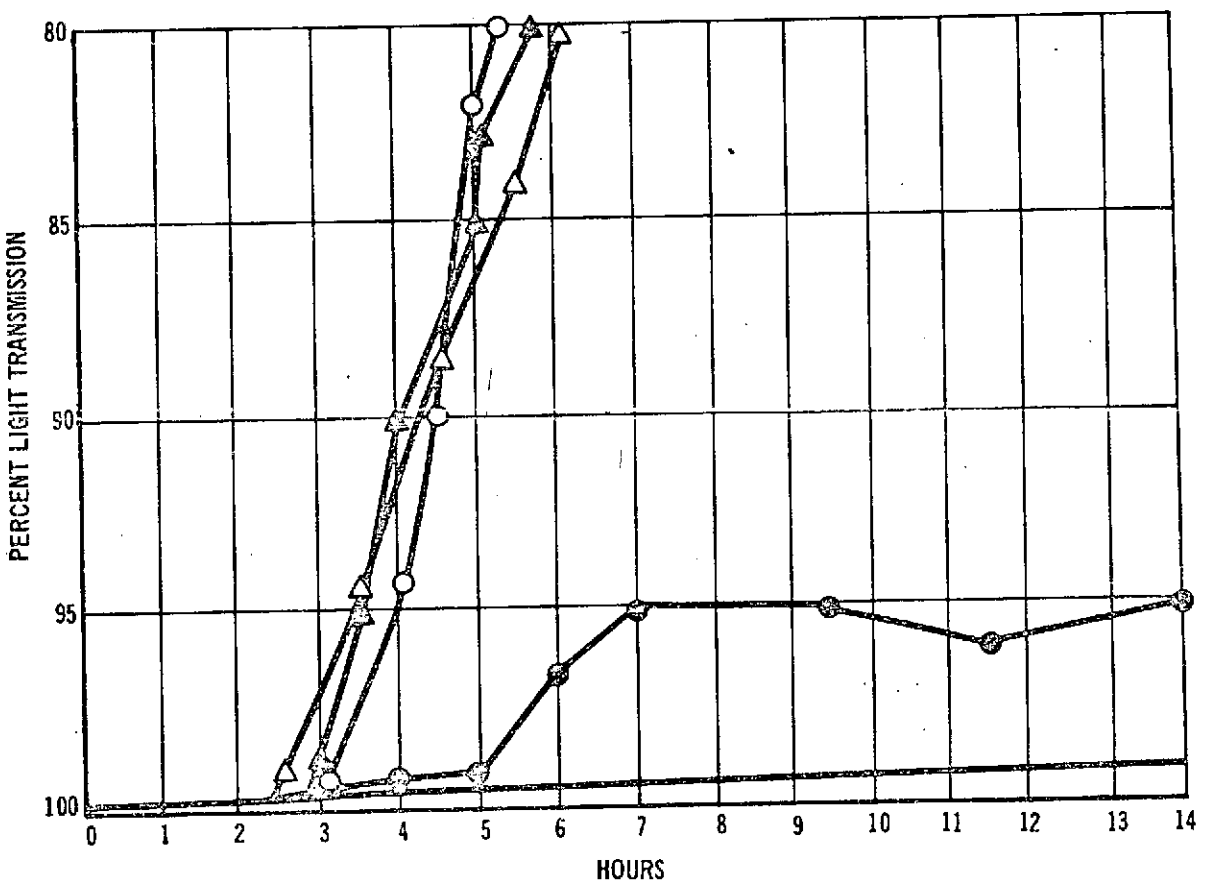
c) We seeded Pseudomonas aeuruginosa into human throat swab inoculum and urine. Figures 39 and 40 presents the results. Inspection of the data reveals system sensitivity of 10 urine organisms and about 1×10^3 for throat. However, the throat specimen tracings seem abnormal and may be an insufficient freeze-dried medium result.

d) We seeded Candida albicans into human throat swab inoculum, urine and feces. Figures 41, 42 and 43 presents results. System sensitivity of this system seems to be between 1×10^2 and 1×10^3 organisms. The control in Figure 41 is special note. We subsequently discovered that the "normal" throat swab contained Candida albicans in small numbers which had been missed on the first clinical lab tests performed. Subsequent same donor throat cultures revealed C. albicans.

e) Escherichia coli was seeded into human feces and throat swab inoculum. Figures 44 and 45 presents our results. Sensitivity varied from 10 organism to 1×10^3.

FIGURE 34
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

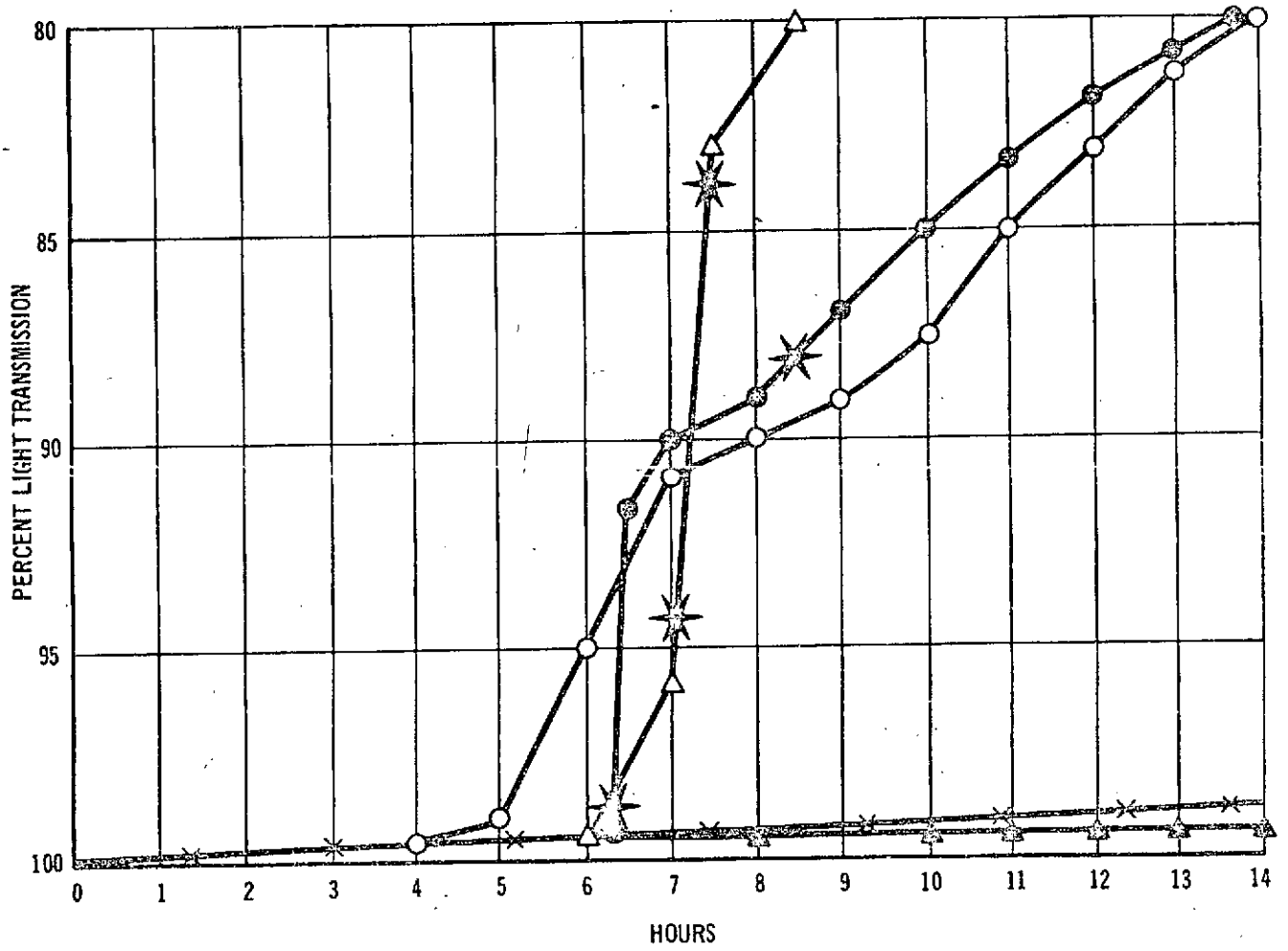
CLINICAL SPECIMEN: HUMAN FECES
TEST MEDIUM: STAPHYLOCOCCUS
SEED ORGANISM: STAPHYLOCOCCUS AUREUS



CONTROL; FECES INOCULUM PLACED IN DETECTION CELL
DETECTION CELL NO. 1; FECES INOCULUM SEEDED WITH APPROX 10^4 STAPH AUREUS
DETECTION CELL NO. 2; FECES INOCULUM SEEDED WITH APPROX 10^3 STAPH AUREUS
DETECTION CELL NO. 3; FECES INOCULUM SEEDED WITH APPROX 10^2 STAPH AUREUS
DETECTION CELL NO. 4; FECES INOCULUM SEEDED WITH APPROX 10^1 STAPH AUREUS

FIGURE 35
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

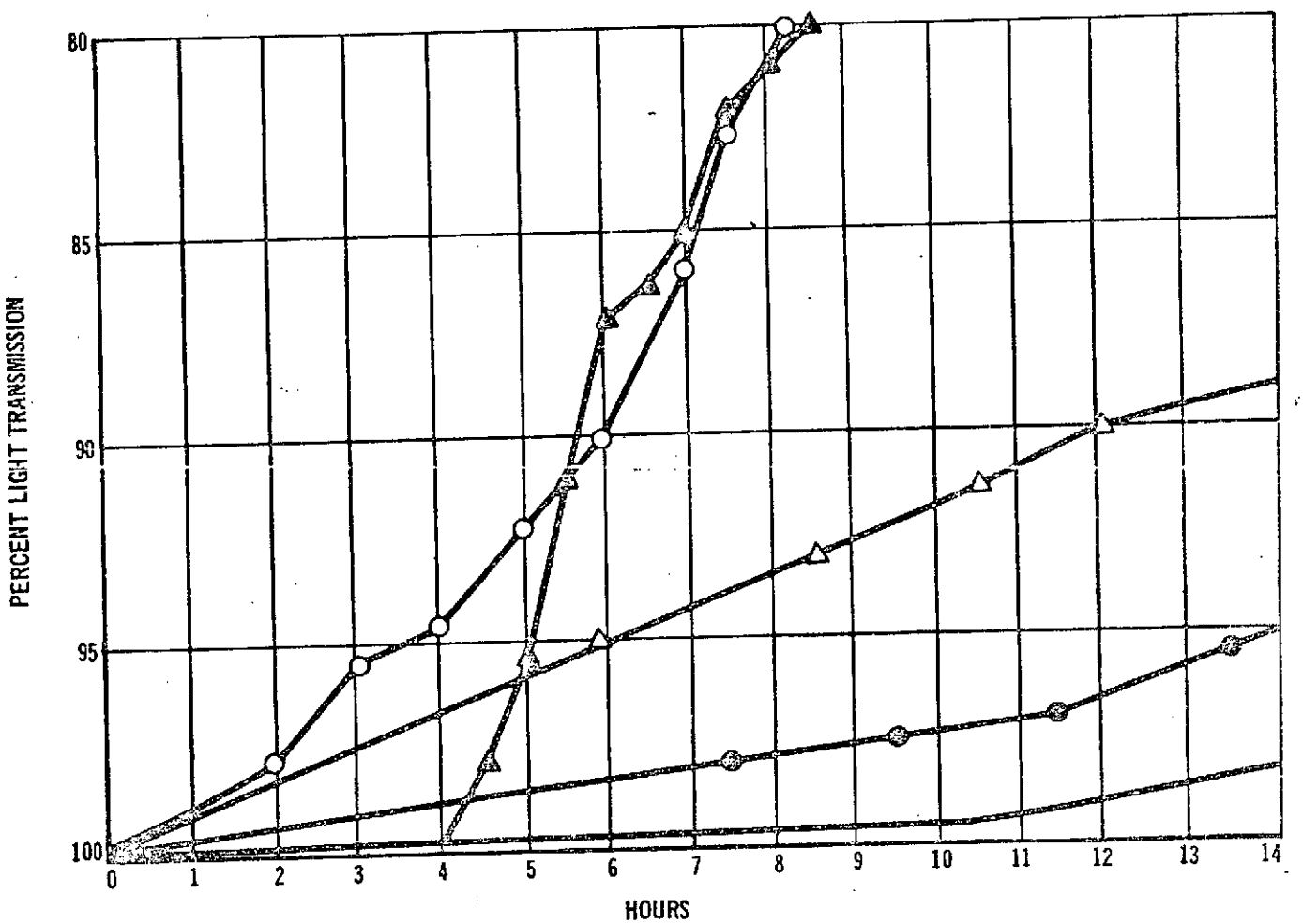
CLINICAL SPECIMEN: HUMAN URINE
TEST MEDIUM: STAPHYLOCOCCUS
SEED ORGANISM: STAPHYLOCOCCUS AUREUS



CONTROL; URINE DILUTED 1/10 PLACED IN DETECTION CELL AS INOCULUM
DETECTION CELL NO. 1; CONTROL SOLUTION SEEDED WITH 10^4 S. AUREUS
DETECTION CELL NO. 2; CONTROL SOLUTION SEEDED WITH 10^3 S. AUREUS
DETECTION CELL NO. 3; CONTROL SOLUTION SEEDED WITH 10^2 S. AUREUS
DETECTION CELL NO. 4; CONTROL SOLUTION SEEDED WITH <10 S. AUREUS
VISUAL INSPECTION REVEALS BUBBLE

FIGURE 36
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

CLINICAL SPECIMEN: THROAT
MEDIUM: STAPHYLOCOCCUS
SEED ORGANISM: STAPHYLOCOCCUS AUREUS



CONTROL; THROAT SWAB INOCULUM PLACED IN DETECTION CELL

DETECTION CELL NO. 1; THROAT INOCULUM SEEDED WITH APPROX 10^4 STAPH AUREUS ORG/ML

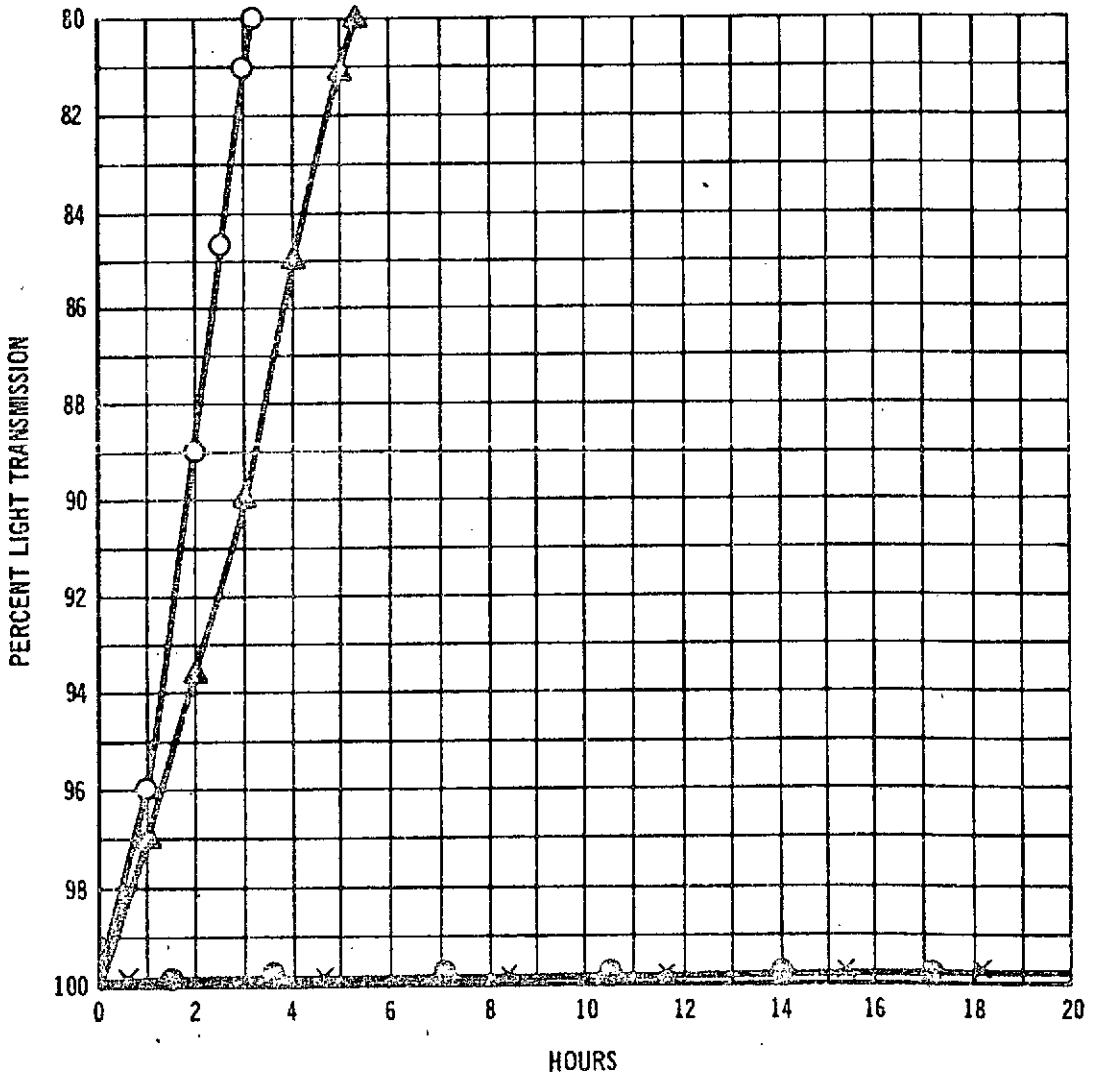
DETECTION CELL NO. 2; THROAT INOCULUM SEEDED WITH APPROX 10^3 STAPH AUREUS ORG/ML

DETECTION CELL NO. 3; THROAT INOCULUM SEEDED WITH APPROX 10^2 STAPH AUREUS ORG/ML

DETECTION CELL NO. 4; THROAT INOCULUM SEEDED WITH APPROX 10^1 STAPH AUREUS ORG/ML

FIGURE 37
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

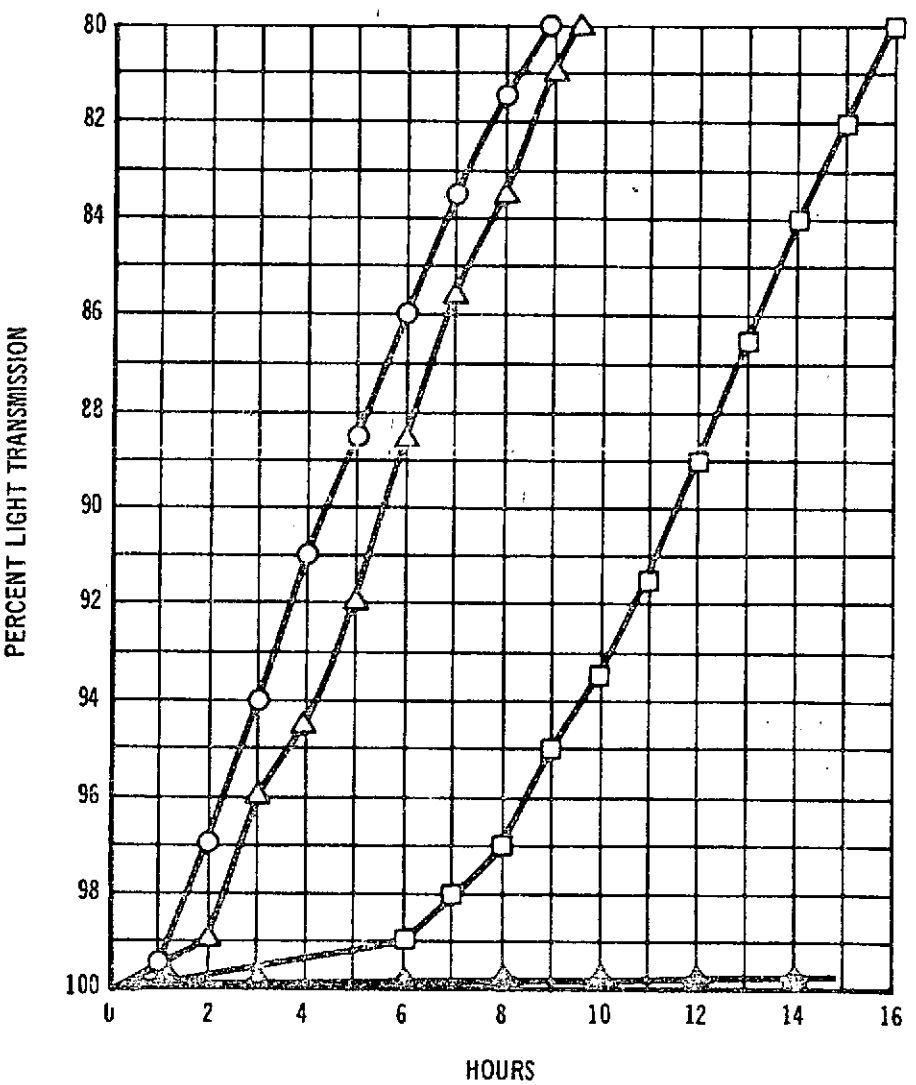
CLINICAL SPECIMEN: THROAT
MEDIUM: STAPHYLOCOCCUS
SEED ORGANISM: STAPHYLOCOCCUS AUREUS



CONTROL; THROAT INOCULUM PLACED IN DETECTION CELL
DETECTION CELL NO. 1; THROAT INOCULUM + S. AUREUS (10^4)
DETECTION CELL NO. 2; THROAT INOCULUM + S. AUREUS (10^3)
DETECTION CELL NO. 3; THROAT INOCULUM + S. AUREUS (10^2)

FIGURE 38
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

CLINICAL SPECIMEN: HUMAN URINE
TEST MEDIUM: SALMONELLA
SEED ORGANISM: SALMONELLA TYPHOSA



△—△ CONTROL; DILUTED URINE PLACED IN DETECTION CELL AS INOCULUM
○—○ DETECTION CELL NO. 1; URINE SEEDED WITH SALMONELLA TYPHOSA (10^4)
△—△ DETECTION CELL NO. 2; URINE SEEDED WITH SALMONELLA TYPHOSA (10^3)
□—□ DETECTION CELL NO. 3; URINE SEEDED WITH SALMONELLA TYPHOSA (10^2)

FIGURE 39
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED
WITH FREEZE-DRIED MEDIA

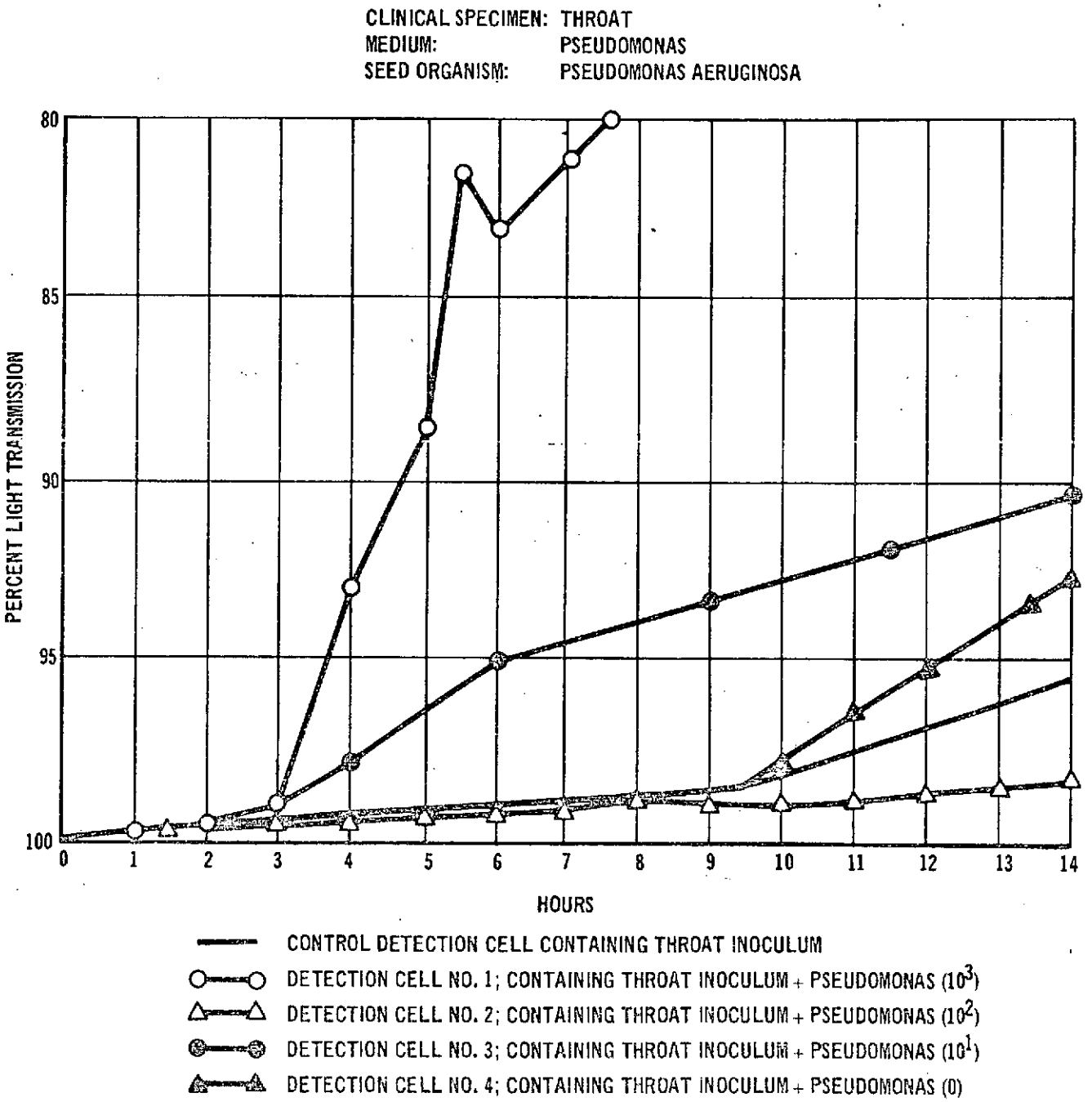


FIGURE 40
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

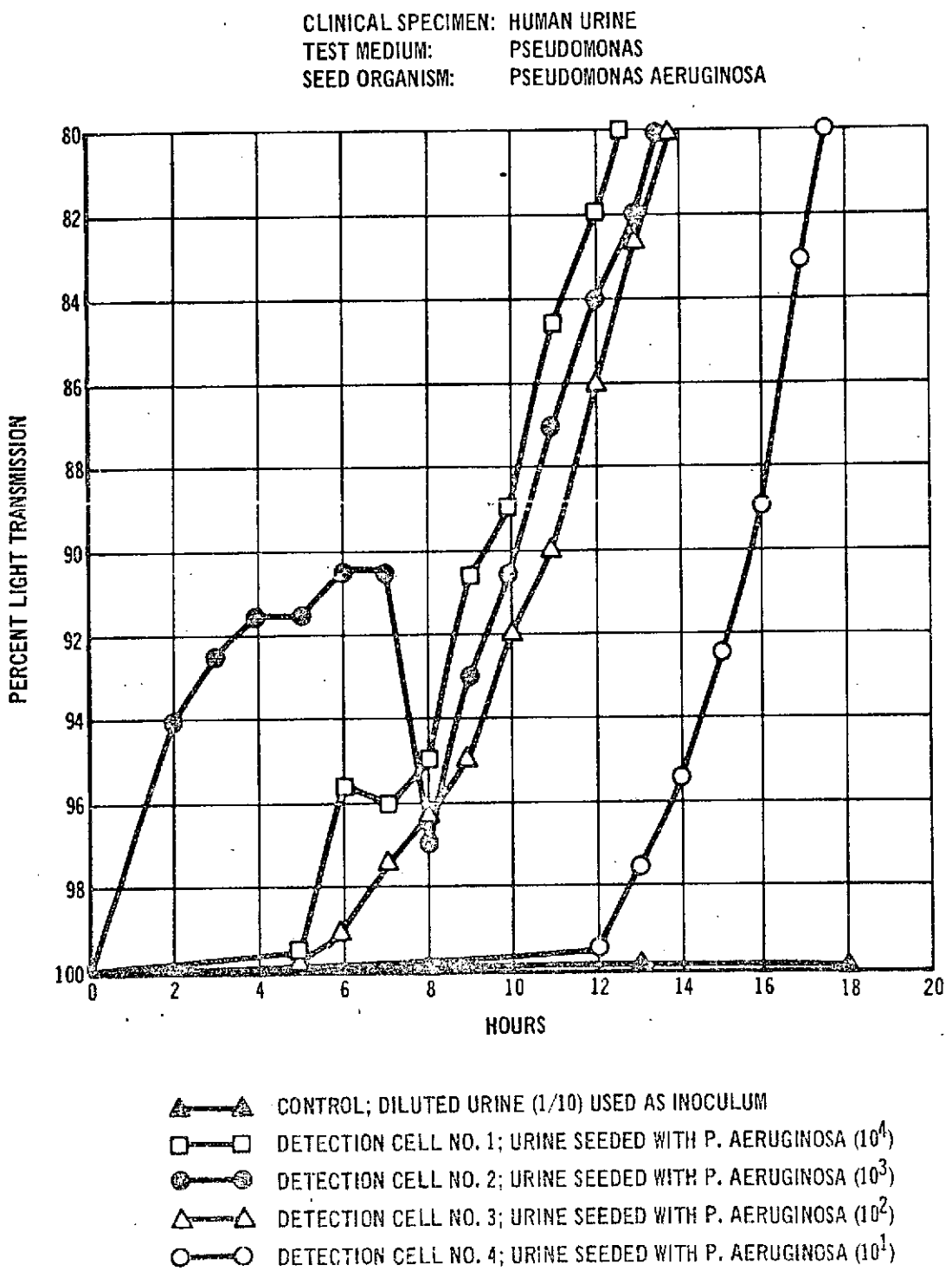
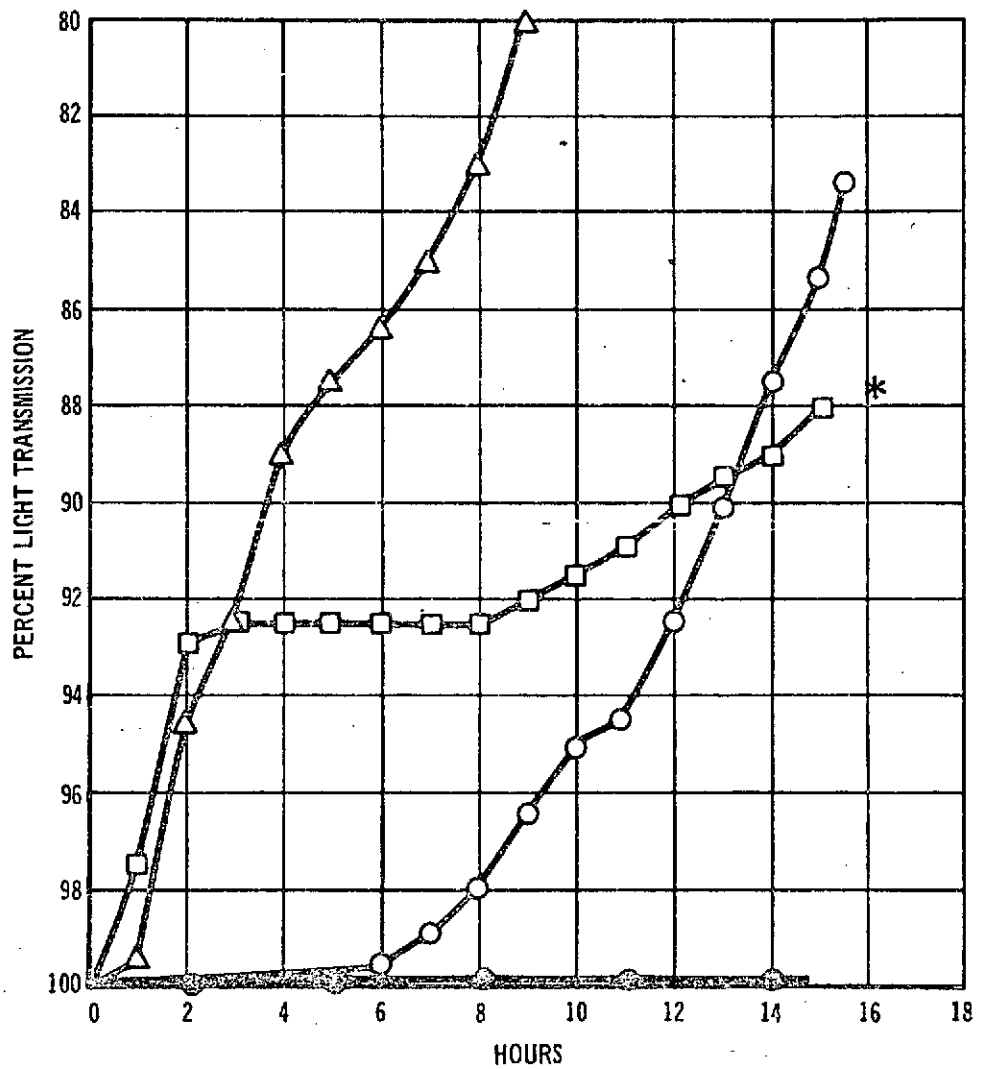


FIGURE 41
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

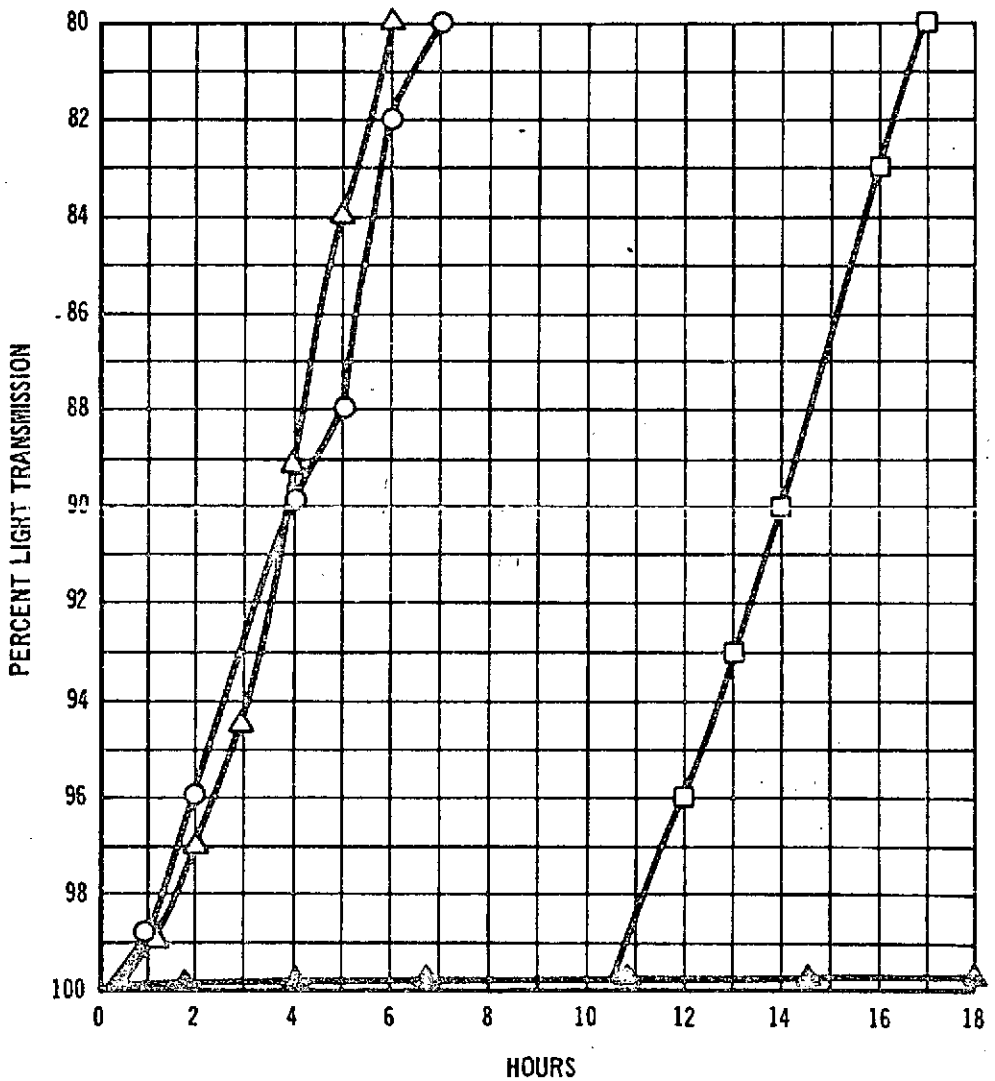
CLINICAL SPECIMEN: THROAT
MEDIUM: CANDIDA
SEED ORGANISM: CANDIDA ALBICANS



CONTROL; THROAT SWAB INOCULUM PLACED IN DETECTION CELL
DETECTION CELL NO. 1; THROAT INOCULUM SEEDED WITH C. ALBICANS (10^4)
DETECTION CELL NO. 2; THROAT INOCULUM SEEDED WITH C. ALBICANS (10^3)
DETECTION CELL NO. 3; THROAT INOCULUM SEEDED WITH C. ALBICANS (10^2)
* CONTROL THROAT SWAB FOUND TO CONTAIN CANDIDA ALBICANS

FIGURE 42
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

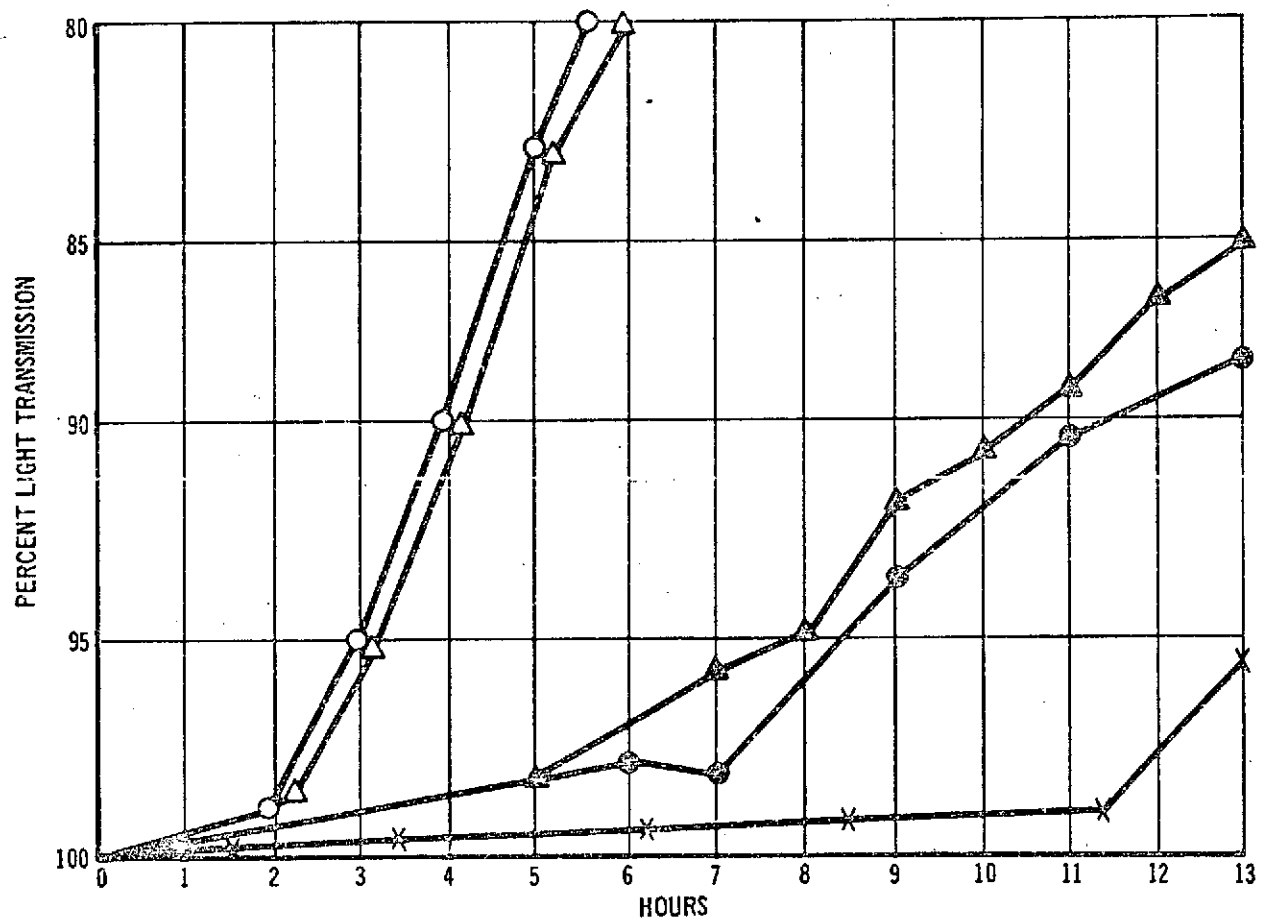
CLINICAL SPECIMEN: HUMAN URINE
TEST MEDIUM: CANDIDA
SEED ORGANISM: CANDIDA ALBICANS



- CONTROL; DILUTED URINE PLACED IN DETECTION CELL AS INOCULUM
- DETECTION CELL NO. 1; URINE SEEDED WITH CANDIDA ALBICANS (10^4)
- DETECTION CELL NO. 2; URINE SEEDED WITH CANDIDA ALBICANS (10^3)
- DETECTION CELL NO. 3; URINE SEEDED WITH CANDIDA ALBICANS (10^2)

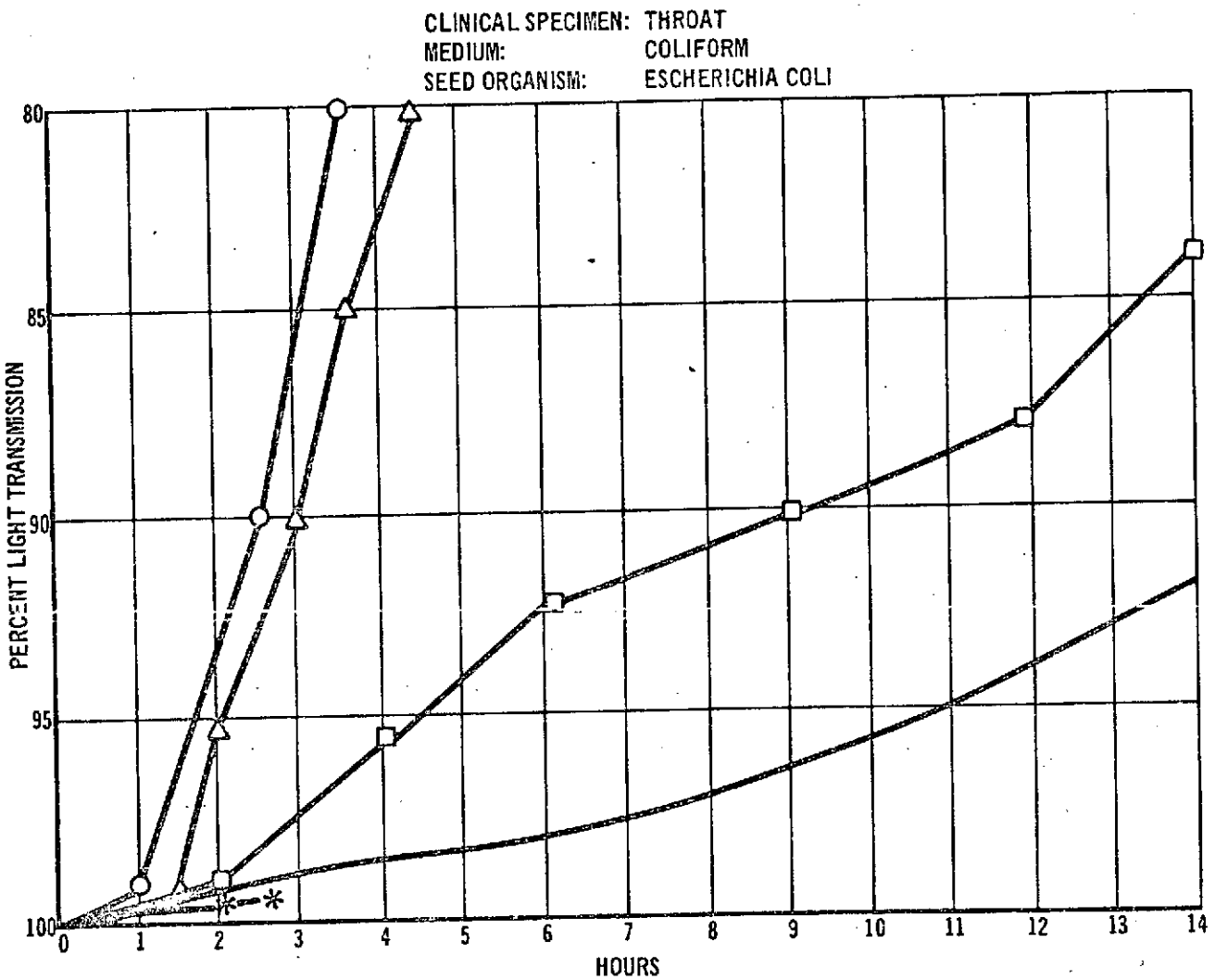
FIGURE 43
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

CLINICAL SPECIMEN: HUMAN FECES
TEST MEDIUM: CANDIDA
SEED ORGANISM: CANDIDA ALBICANS



CONTROL; FECES DILUTED AND PLACED IN DETECTION CELL AS INOCULUM TUBE
DETECTION CELL NO. 1; FECES INOCULUM SEEDED WITH APPROX 10^4 C. ALBICANS
DETECTION CELL NO. 2; FECES INOCULUM SEEDED WITH APPROX 10^3 C. ALBICANS
DETECTION CELL NO. 3; FECES INOCULUM SEEDED WITH APPROX 10^2 C. ALBICANS
DETECTION CELL NO. 4; FECES INOCULUM SEEDED WITH APPROX 10^1 C. ALBICANS

FIGURE 44
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA



CONTROL; THROAT SWAB INOCULUM IN DETECTION CELL

DETECTION CELL NO. 1; THROAT SPECIMEN SEEDED WITH APPROX 10^5 E. COLI ORG/ML

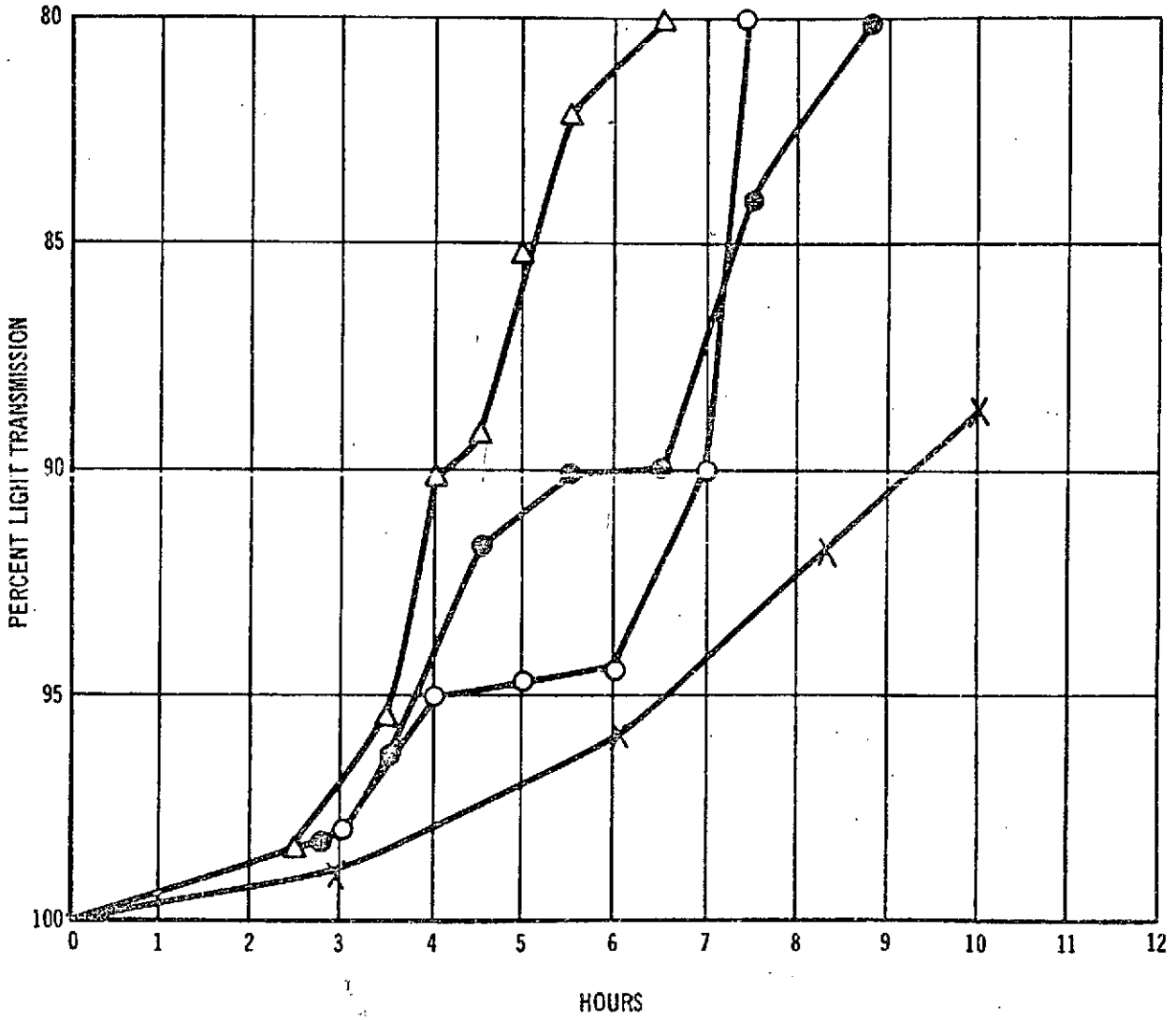
DETECTION CELL NO. 2; THROAT SPECIMEN SEEDED WITH APPROX 10^4 E. COLI ORG/ML

DETECTION CELL NO. 3; THROAT SPECIMEN SEEDED WITH APPROX 10^3 E. COLI ORG/ML

DETECTION CELL NO. 4; ELECTRONIC MALFUNCTION

FIGURE 45
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

CLINICAL SPECIMEN: HUMAN FECES
TEST MEDIUM: COLIFORM
BEST ORGANISM: ESCHERICHIA COLI



CONTROL; FECES INOCULUM PLACED IN DETECTION CELL
DETECTION CELL NO. 1; FECES INOCULUM SEEDED WITH APPROX 10^3 E. COLI
DETECTION CELL NO. 2; FECES INOCULUM SEEDED WITH APPROX 10^2 E. COLI
DETECTION CELL NO. 3; FECES INOCULUM SEEDED WITH APPROX 10^1 E. COLI

f) Proteus morganii was seeded into human urine, feces, and throat inoculum. Our results are in Figures 46, 47, 48 and 49. All specimens' sensitivity was approximately 1×10^2.

g) Aspergillus niger was seeded into an ear swab inoculum. Results are presented in Figure 50. Sensitivity was approximately 1×10^3 spores.

h) Diplococcus pneumoniae was seeded into throat swab inoculum. Results are presented in Figure 51. Sensitivity of this system requires 1×10^3 to 1×10^4 D. pneumoniae.

i) Mima polymorpha was seeded into inoculum from a human skin swab. Figure 52 presents the results. Sensitivity required presence of approximately 1×10^3 organisms.

j) Beta hemolytic Streptococcus, type A, was seeded into human throat swab inoculum. Red blood cells were supplied at the time of cassette filling. Figure 53 depicts the results. The control detection chamber had measurable hemolysis due to large Staphylococcus aureus numbers which, although not growing, were metabolically active enough to produce measurable hemolysis.

k) Herellea vaginicola was seeded into inocolum from human skin. Results are presented in Figure 54. Sensitivity of the system required the presence of at least 1×10^3 organisms.

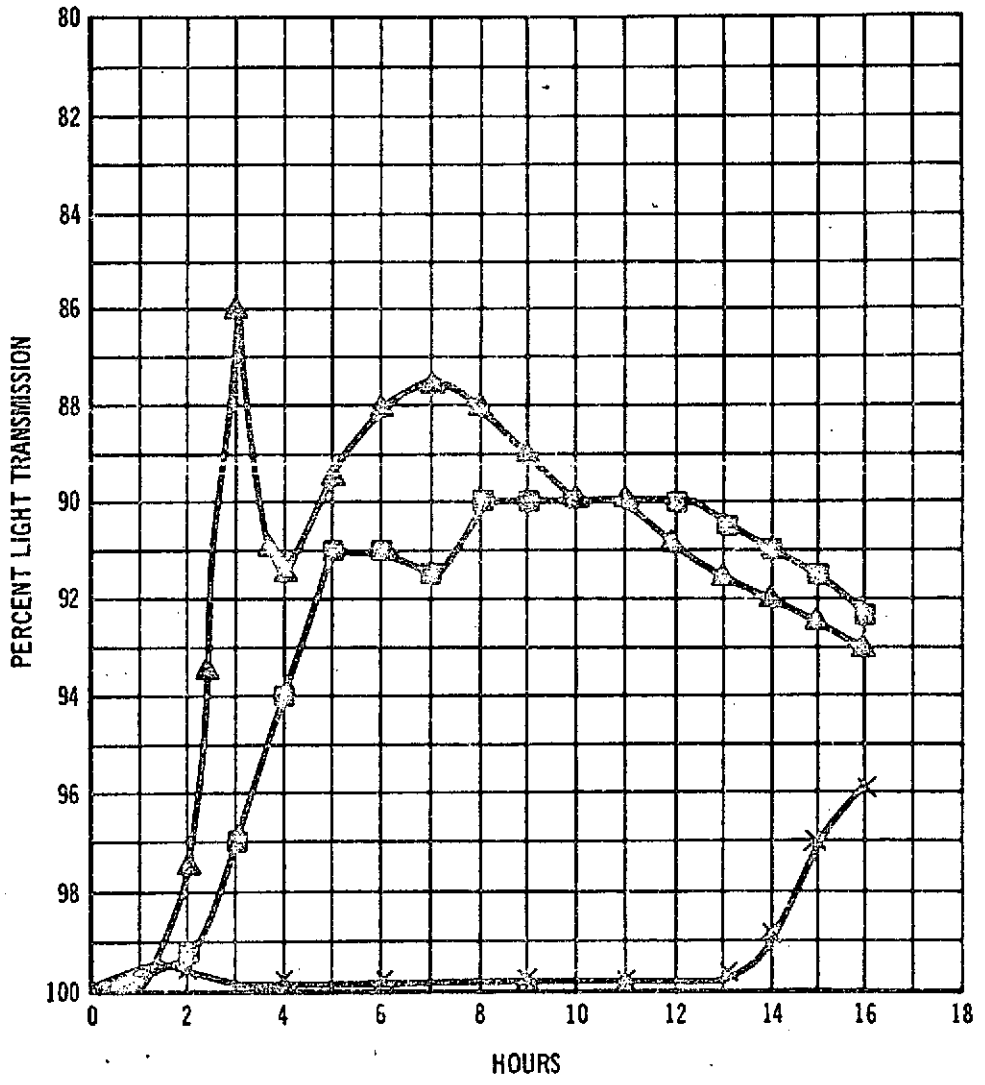
l) Neisseria meningiditis was seeded into inoculum from a throat culture. Figure 55 is the results. Sensitivity was 1×10^2 organisms, however, full scale detector deflection was not observed for 20 hours. Full scale deflection was observed in 13 hours with a 1×10^4 organism inoculum.

In all of the above data, inoculum size refers to the calculated organism number present in the fluid between consecutive filters. This fluid volume includes the detection cell viewed by the MLM.

3.5.4 Completed Evaluation of MLM Media - The total MLM system success depends upon the freeze-dried media ability to extend the preprogrammed cassettes' shelf life. In addition, we deemed it desirable to freeze and store the entire filter cassette after MLM detection, so that organisms of interest can be

FIGURE 46
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

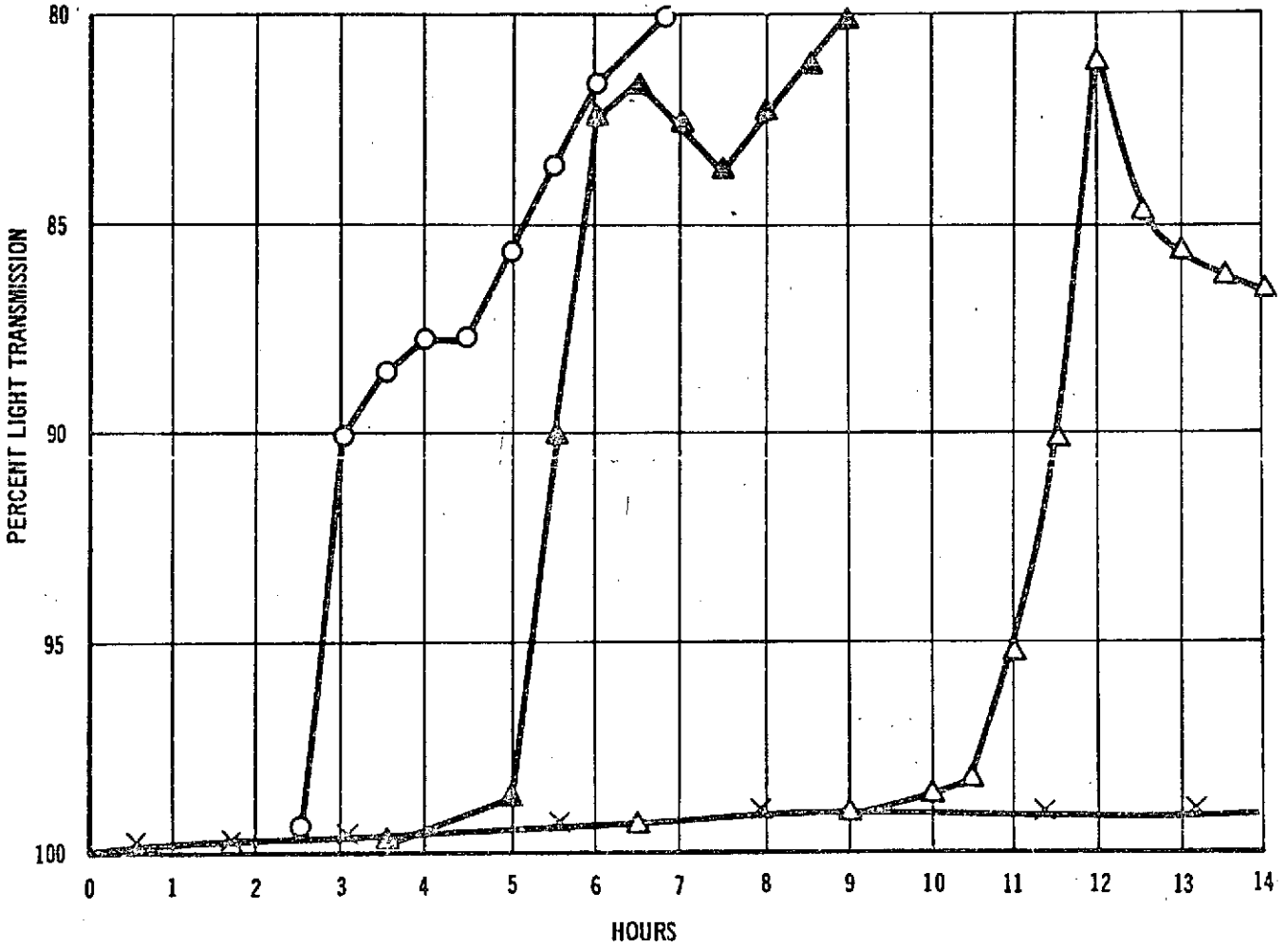
CLINICAL SPECIMEN: HUMAN URINE
TEST MEDIUM: UREASE
SEED ORGANISM: PROTEUS MIRABILIS



CONTROL; URINE DILUTED 1/10 PLACED IN DETECTION CELL AS INOCULUM
DETECTION CELL NO. 1; URINE INOCULUM SEEDED WITH P. MIRABILIS (10^4)
DETECTION CELL NO. 2; URINE INOCULUM SEEDED WITH P. MIRABILIS (10^3)

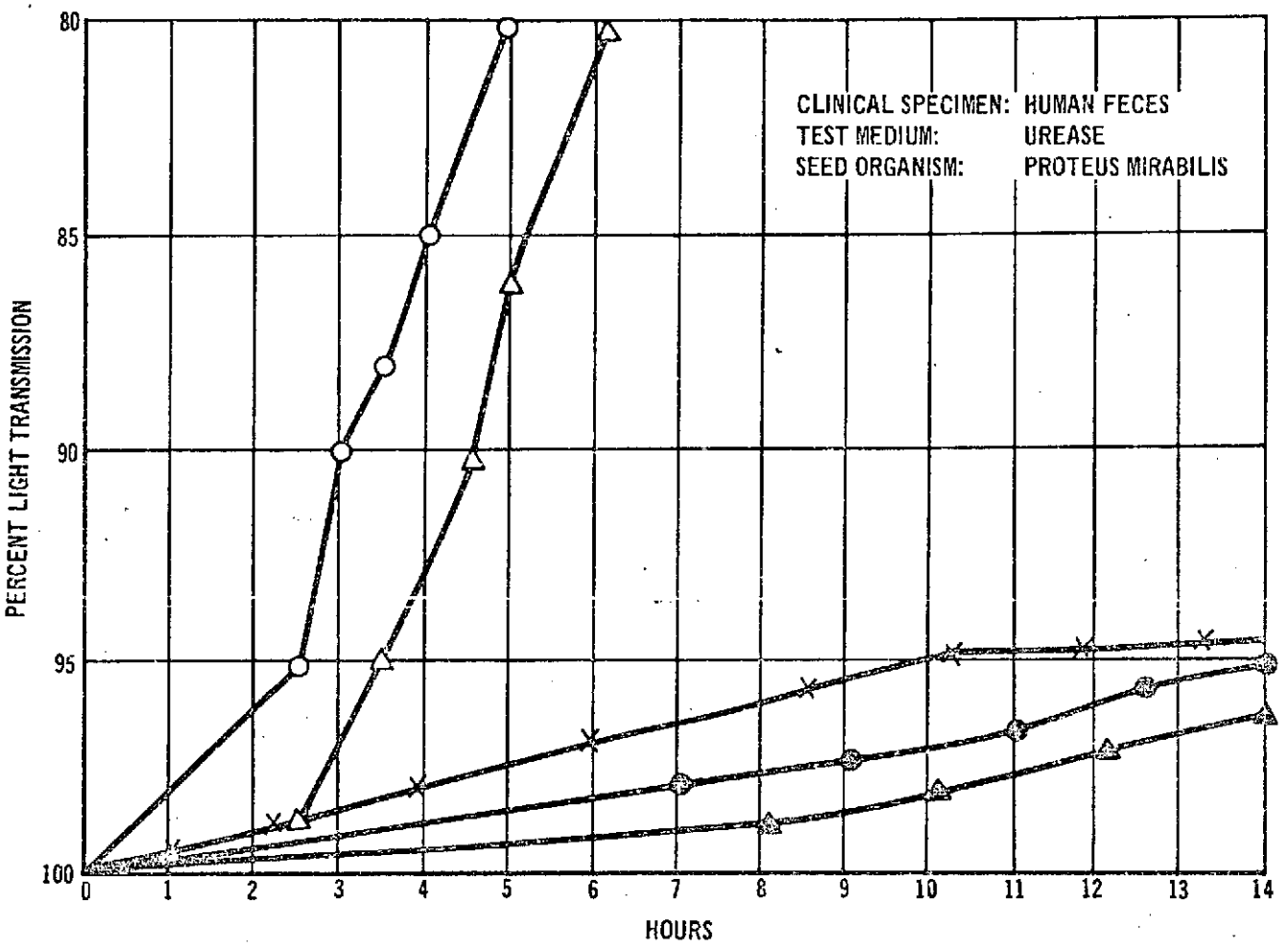
FIGURE 47
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

CLINICAL SPECIMEN: HUMAN URINE
TEST MEDIUM: UREASE
SEED ORGANISM: PROTEUS MIRABILIS



CONTROL; HUMAN URINE DILUTED 1/10 PLACED IN DETECTION CELL AS INOCULUM
DETECTION CELL NO. 1; URINE INOCULUM SEEDED WITH P. MIRABILIS (10^4)
DETECTION CELL NO. 2; URINE INOCULUM SEEDED WITH P. MIRABILIS (10^3)
DETECTION CELL NO. 3; URINE INOCULUM SEEDED WITH P. MIRABILIS (10^2)

FIGURE 48
MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED
WITH FREEZE-DRIED MEDIA



CONTROL; FECES PLACED IN DETECTION CELL AS INOCULUM
DETECTION CELL NO. 1; FECES SEEDED WITH APPROX 10^4 P. MIRABILIS
DETECTION CELL NO. 2; FECES SEEDED WITH APPROX 10^3 P. MIRABILIS
DETECTION CELL NO. 3; FECES SEEDED WITH APPROX $< 10^2$ P. MIRABILIS
DETECTION CELL NO. 4; FECES SEEDED WITH APPROX 10^1 P. MIRABILIS

FIGURE 49

MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

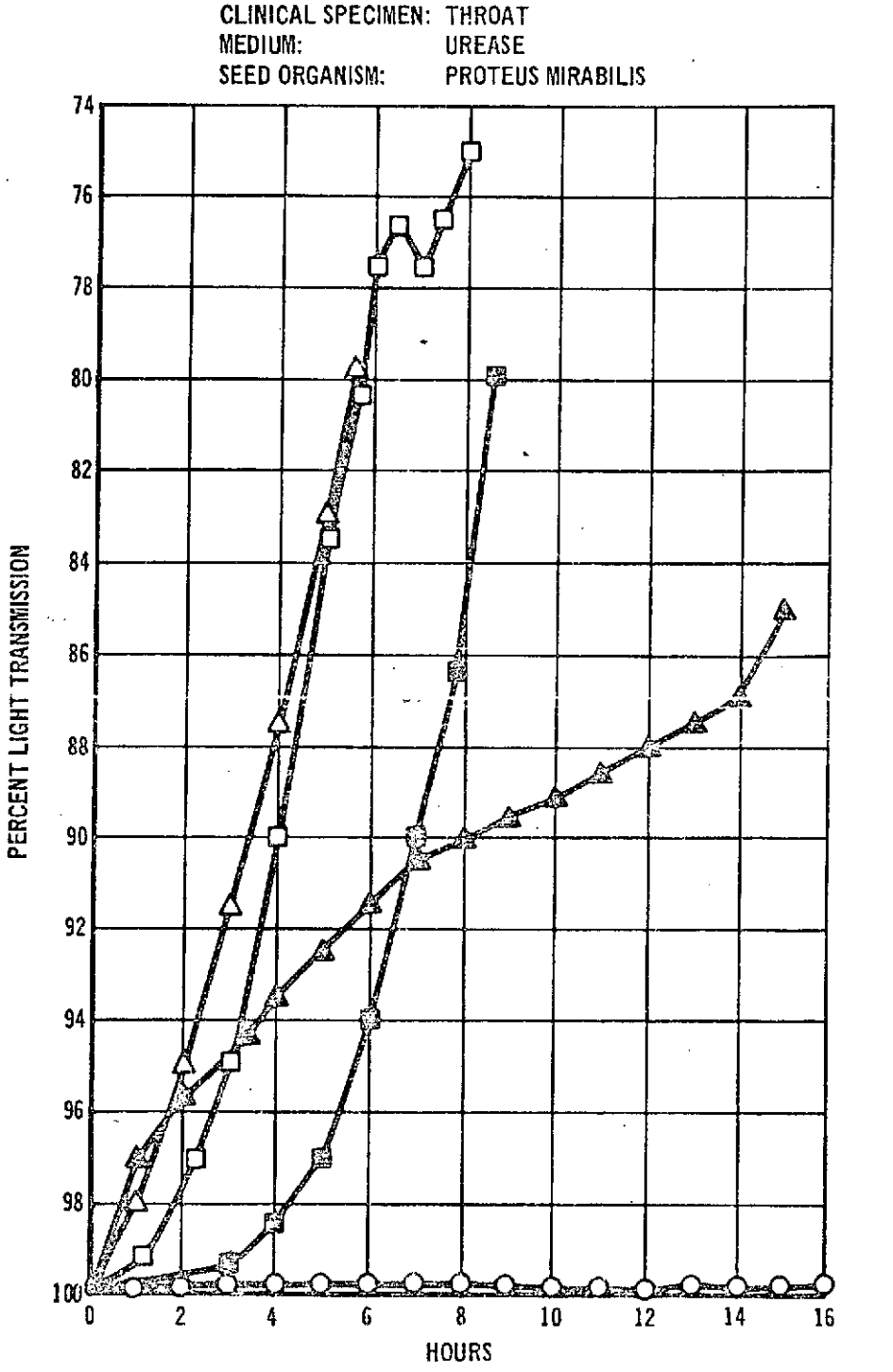
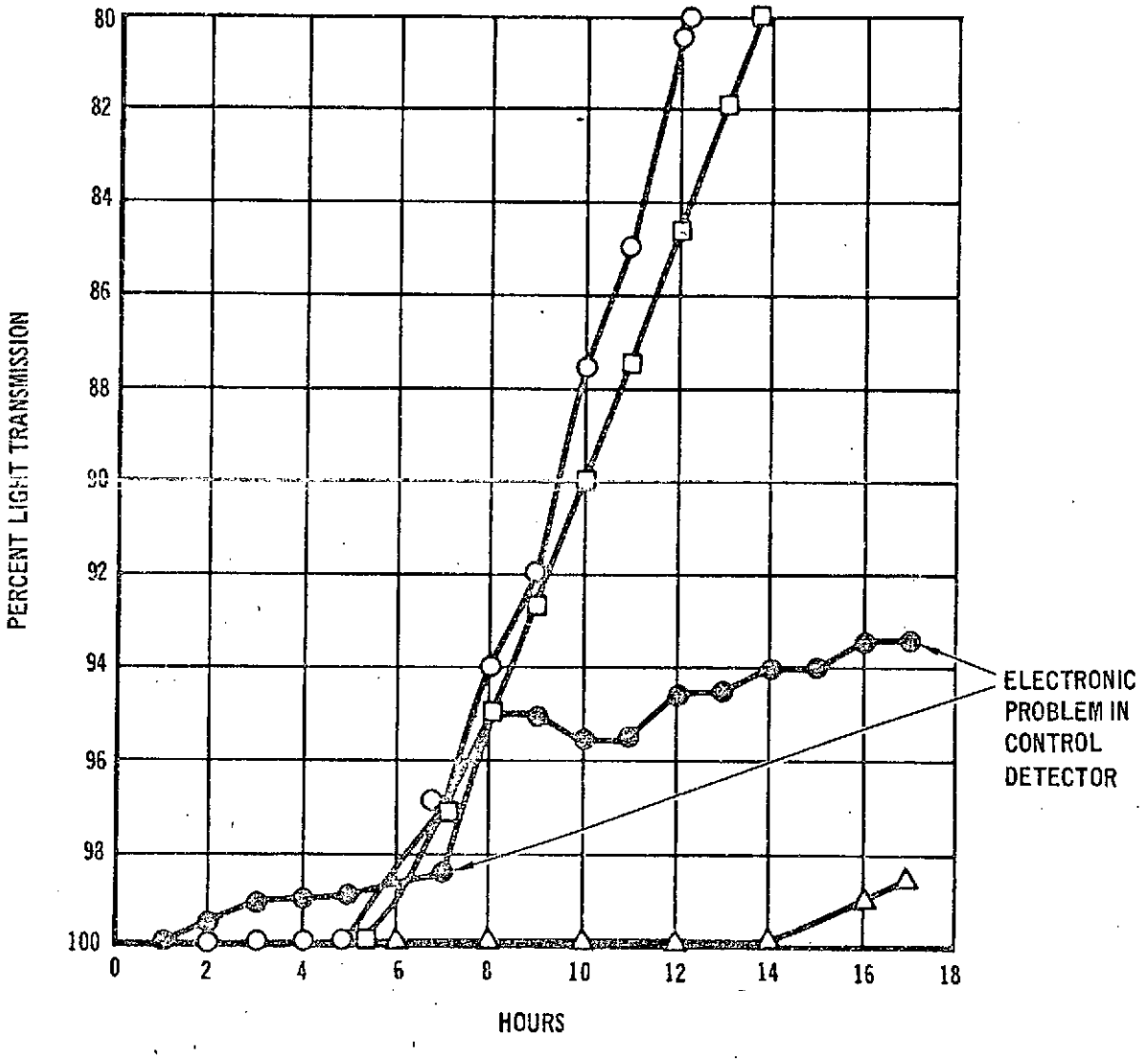


FIGURE 50

MLM STUDIES WITH FILTER CASSETTES PRE-PROGRAMMED WITH FREEZE-DRIED MEDIA

CLINICAL SPECIMEN: EAR SWAB
TEST MEDIUM: ASPERGILLUS MEDIUM
SEED ORGANISM: SPORES OF ASPERGILLUS NIGER



CONTROL; DETECTION CELL INOCULATED WITH EAR SWAB

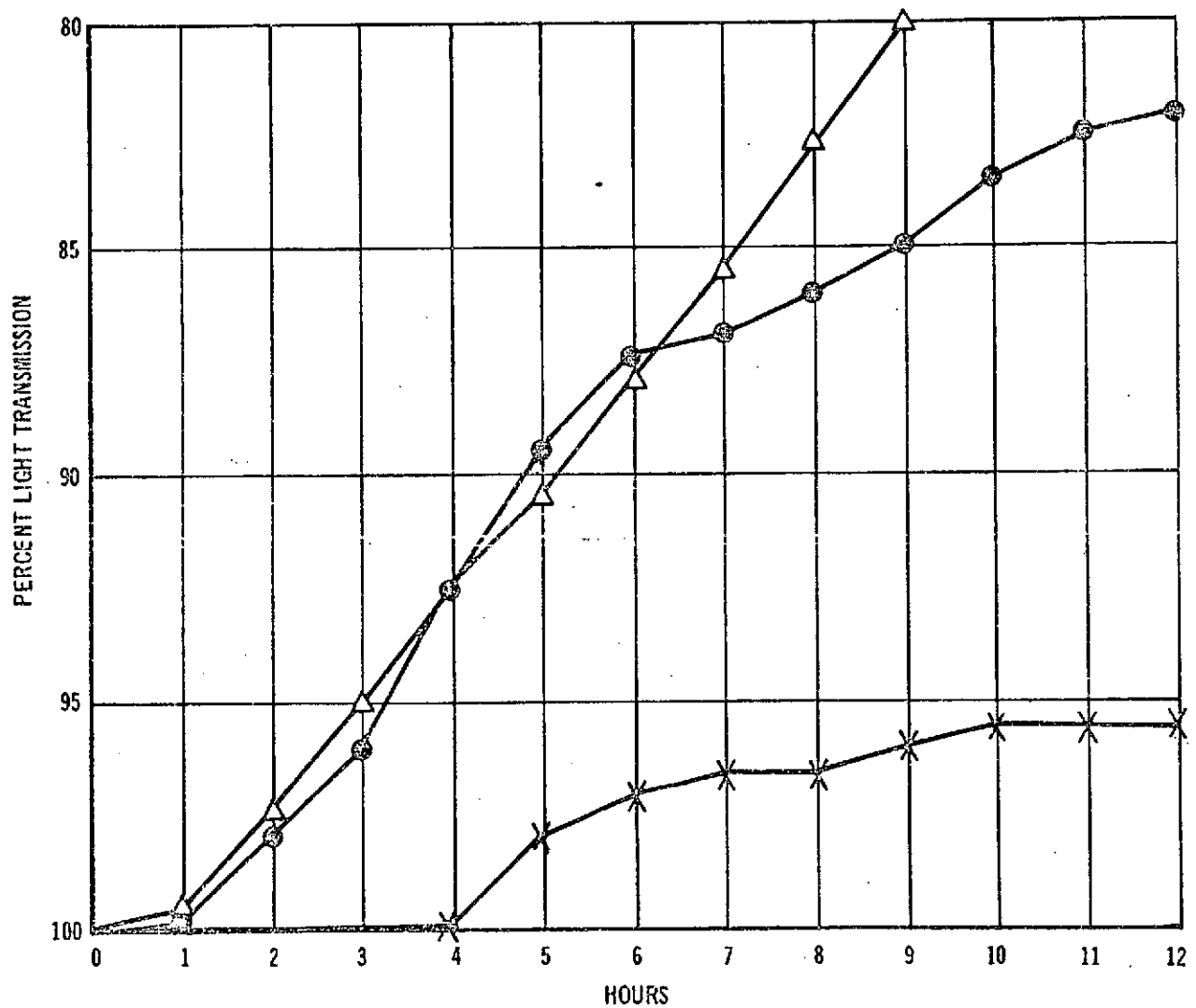
DETECTION CELL NO. 1, EAR SWAB INOCULUM SEEDED WITH A. NIGER SPORES (10^4)

DETECTION CELL NO. 2, EAR SWAB INOCULUM SEEDED WITH A. NIGER SPORES (10^3)

DETECTION CELL NO. 3, EAR SWAB INOCULUM SEEDED WITH A. NIGER SPORES (10^2)

FIGURE 51
MLM STUDIES WITH FILTER CASSETTES

CLINICAL SPECIMEN: THROAT SWAB
MEDIUM: INULIN SERUM BROTH
SEED ORGANISMS: DIPLOCOCCUS PNEUMONIAE



CONTROL; THROAT SWAB INOCULUM PLACED IN DETECTION CELL

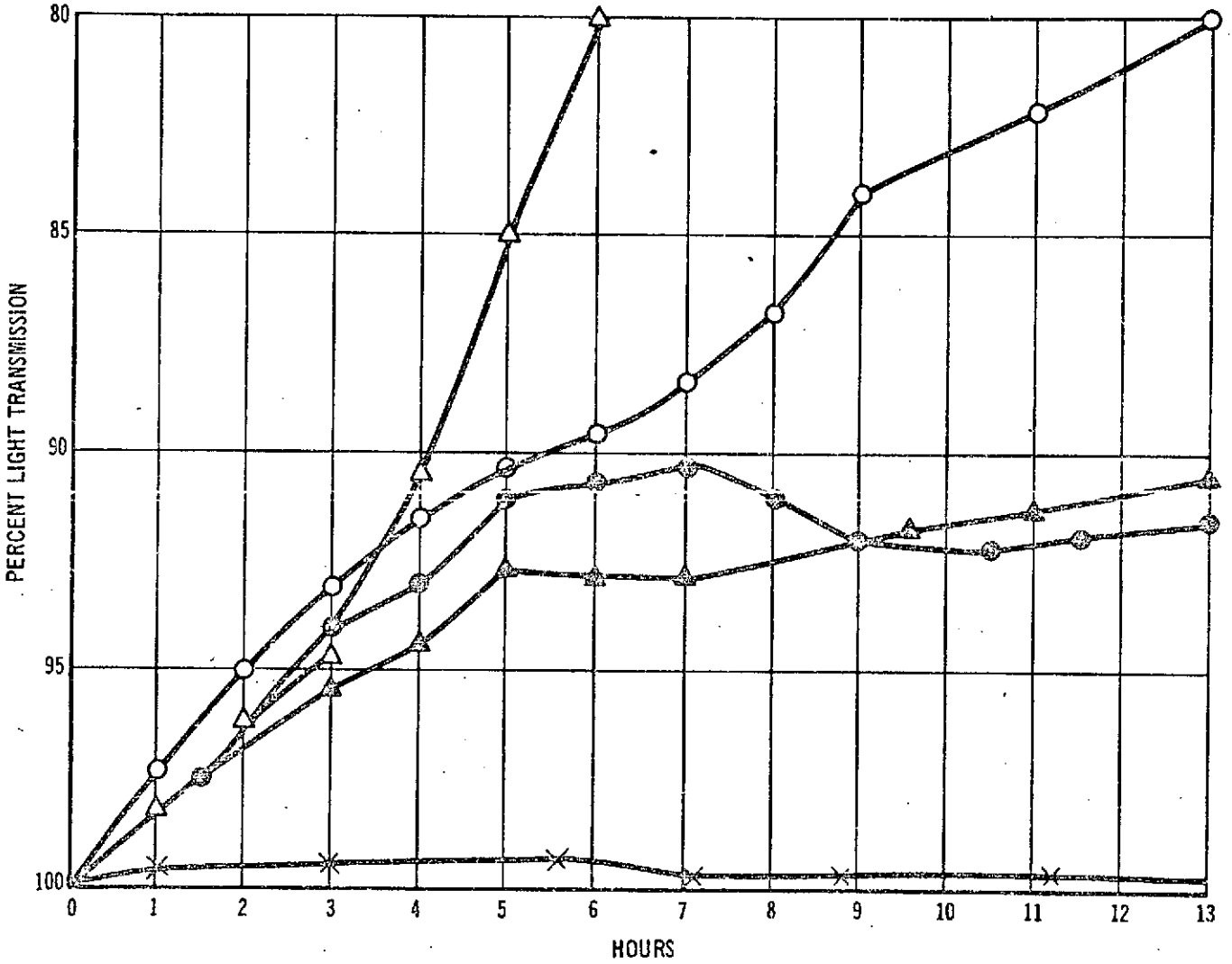
DETECTION CELL NO. 2; THROAT SWAB SEEDED WITH APPROX 10^4 D. PNEUMONIAE

DETECTION CELL NO. 3; THROAT SWAB SEEDED WITH APPROX 10^3 D. PNEUMONIAE

FIGURE 52

MLM STUDIES WITH FILTER CASSETTES

CLINICAL SPECIMEN: SKIN SWAB
MEDIUM: MIMA MEDIUM
SEED ORGANISM: MIMA POLYMORPHA



CONTROL; SKIN SWAB INOCULUM PLACED IN DETECTION CELL

DETECTION CELL NO. 1; SKIN SWAB SEEDED WITH APPROX 10^4 M. POLYMORPHA

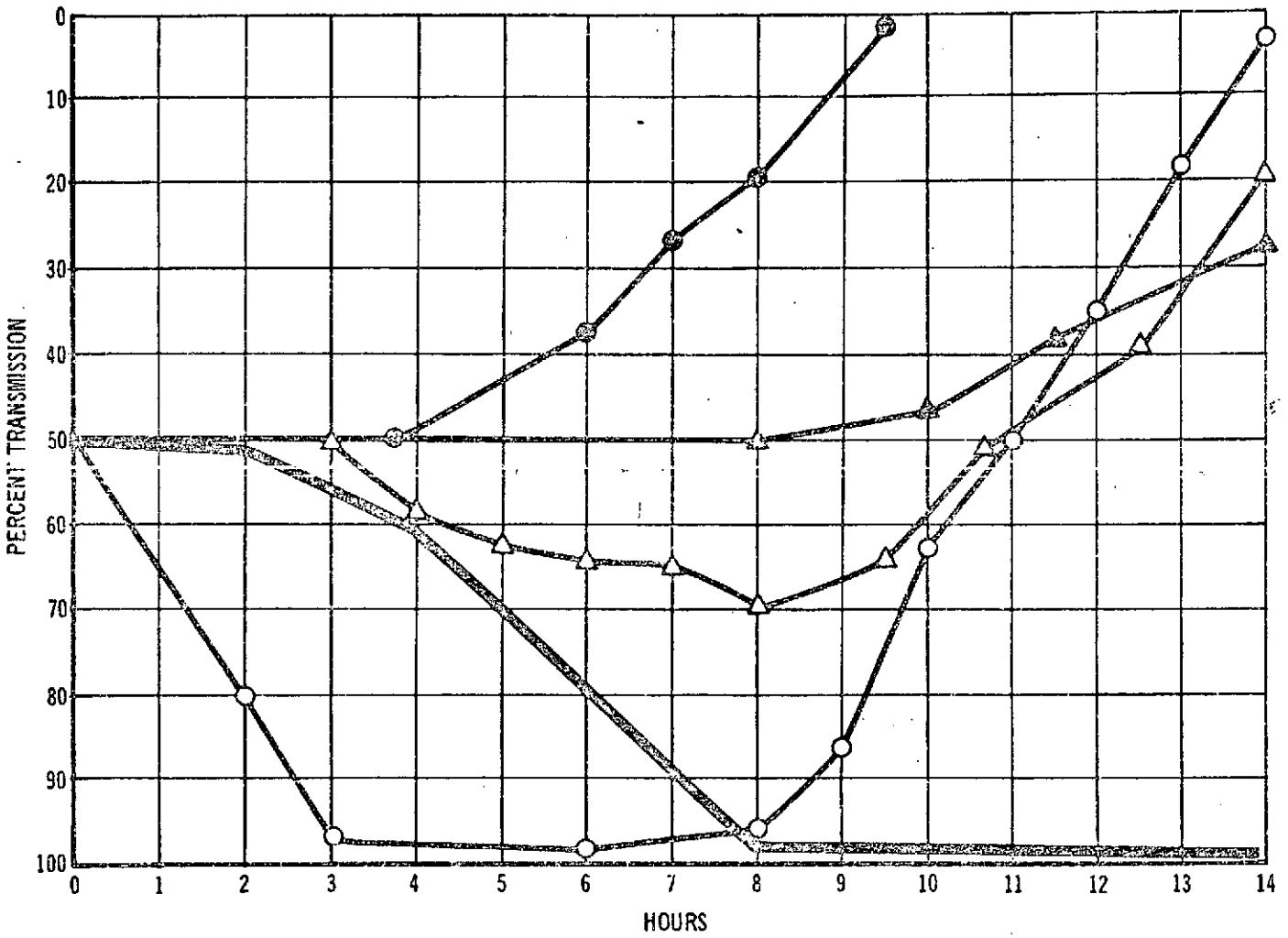
DETECTION CELL NO. 2; SKIN SWAB SEEDED WITH APPROX 10^3 M. POLYMORPHA

DETECTION CELL NO. 3; SKIN SWAB SEEDED WITH APPROX 10^2 M. POLYMORPHA

DETECTION CELL NO. 4; SKIN SWAB SEEDED WITH APPROX 10^1 M. POLYMORPHA

FIGURE 53
MLM STUDIES WITH FILTER CASSETTES

CLINICAL SPECIMEN: THROAT SWAB
TEST MEDIUM: STREPTOCOCCUS MEDIUM WITH RBC
SPEED ORGANISM: TYPE A BETA HEMOLYTIC STREPTOCOCCUS



CONTROL; THROAT SWAB PLACED IN 3 ML H_2O → PLATED ON BLOOD AGAR (RESULTS; 10^3 STAPHYLOCOCCI SP., 10^3 GAMMA STREPTOCOCCI), 1 ML OF ORIGINAL THROAT SUSPENSION → 9 ML MEDIUM PLUS WASHED RBC, 0.3 ML OF THIS SUSPENSION PLACED IN DETECTION CELL

DETECTION CELL NO. 1; THROAT INOCULUM SEEDED WITH APPROX 10^4 β. STREPTOCOCCUS

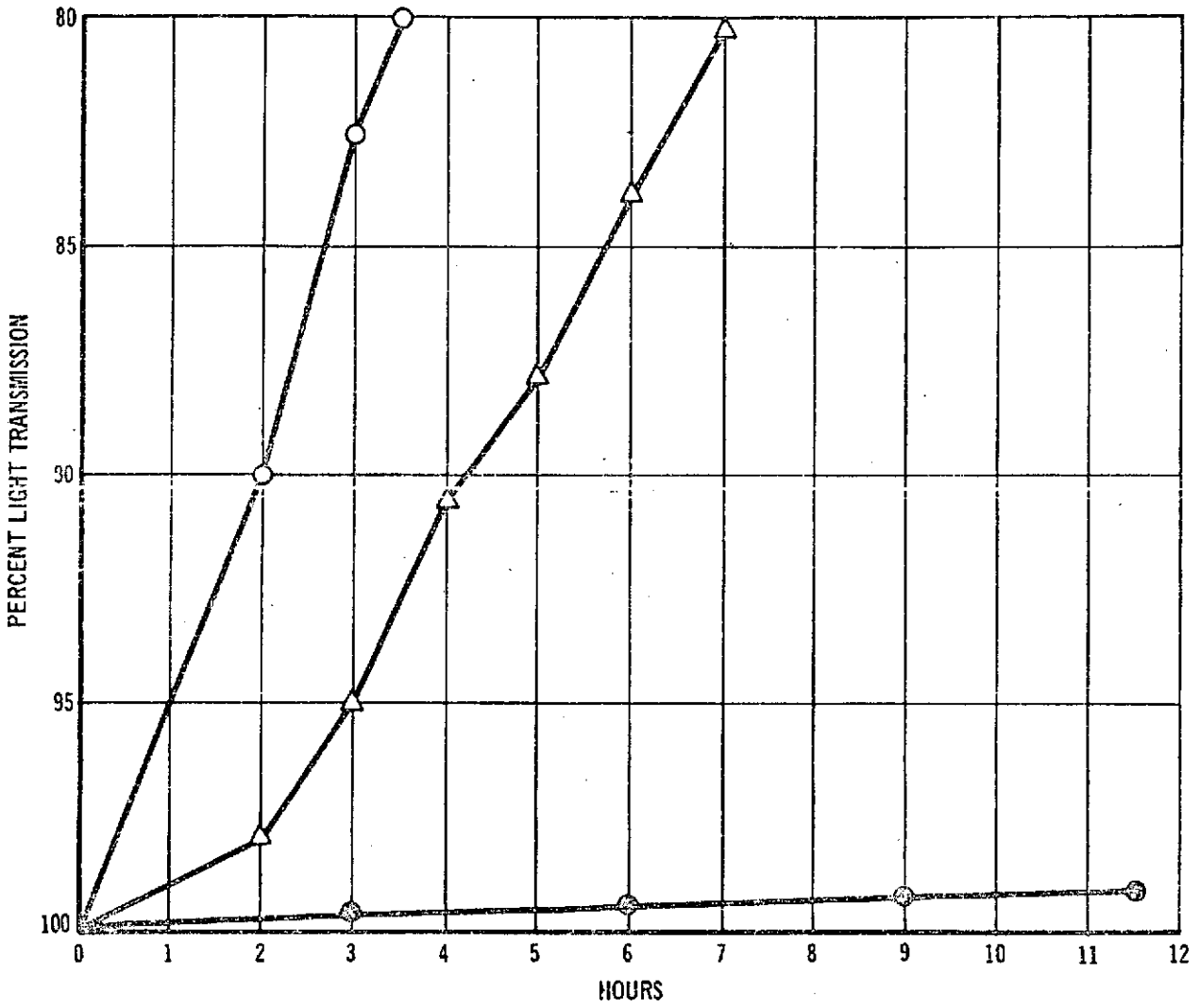
DETECTION CELL NO. 2; THROAT INOCULUM SEEDED WITH APPROX 10^3 β. STREPTOCOCCUS

DETECTION CELL NO. 3; THROAT INOCULUM SEEDED WITH APPROX 10^2 β. STREPTOCOCCUS

DETECTION CELL NO. 4; THROAT INOCULUM SEEDED WITH APPROX 10^0 β. STREPTOCOCCUS

FIGURE 54
MLM STUDIES WITH FILTER CASSETTES

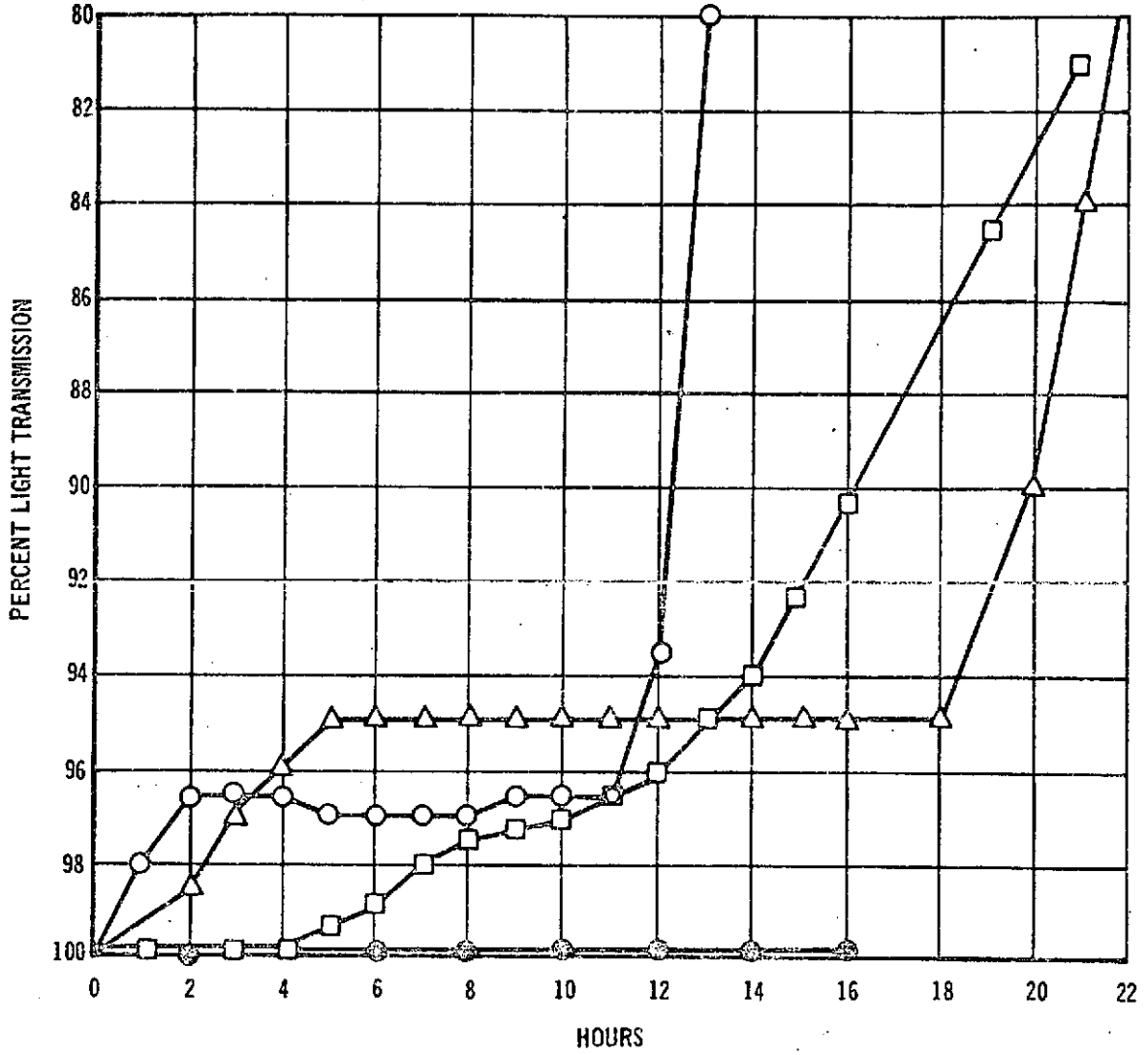
CLINICAL SPECIMEN: HUMAN SKIN SWAB
TEST MEDIUM: HERELLEA BROTH
SEED ORGANISM: HERELLEA VAGINICOLA



DETECTION CELL NO. 1; SKIN SWAB INOCULUM SEEDED WITH APPROX 10^4 H. VAGINICOLA
DETECTION CELL NO. 2; SKIN SWAB INOCULUM SEEDED WITH APPROX 10^3 H. VAGINICOLA
DETECTION CELL NO. 3; SKIN SWAB INOCULUM SEEDED WITH APPROX 10^2 H. VAGINICOLA

FIGURE 55
MLM STUDIES WITH FILTER CASSETTES

CLINICAL SPECIMEN: THROAT
TEST MEDIUM: NEISSERIA BROTH
TEST ORGANISM: NEISSERIA MENINGITIDIS



CONTROL; THROAT SWAB INOCULUM SEEDED WITH N. MENINGITIDIS
DETECTION CELL NO. 1; THROAT SWAB INOCULUM SEEDED WITH N. MENINGITIDIS (10^4)
DETECTION CELL NO. 2; THROAT SWAB INOCULUM SEEDED WITH N. MENINGITIDIS (10^3)
DETECTION CELL NO. 3; THROAT SWAB INOCULUM SEEDED WITH N. MENINGITIDIS (10^2)

studied further several weeks later. These study results are as follows:

a) Shelf life study results on freeze-dried media are presented in Table 27. These data prove that the freeze-dried media shelf life can be at least 4 months, and is probably much longer because no selectivity diminution was observed.

b) Results of freezing the organisms after incubating 16 hours in the appropriate MLM culture broth are presented in Table 28. These data reveal that -79°C storage for 30 days is feasible for all organisms tested. However, Proteus species and Salmonella typhosa were not viable after 60 days storage, while all other test organisms were still viable. Staphylococcus aureus and Candida albicans populations survived the 60 day storage undiminished.

c) Revised MLM Media - Our studies with the complete system suggested some minor culture media changes. For example, the freeze-drying requirement introduces problems with the pH adjustment. Those media normally pH adjusted by adding a volatile acid may lose the acid during the vacuum drying process unless proper buffering is added.

Final tested media formulae in this report are:

STAPHYLOCOCCUS MEDIUM

Beef extract	1g/L
Polypeptone Peptone	10g/L
NaCl	75g/L
D-Mannitol	10g/L
K_2HPO_4	1g/L
1% KTe (Potassium Tellurite)	1 ml/L
Egg Yolk	70g/L

Mix all ingredients except KTe and egg yolk in 930 ml distilled H_2O. Autoclave 15 pounds, 15 minutes. Add KTe and egg yolk to cooled mixture. Adjust pH with phosphates (1/15 M) to 7.2.

TABLE 27
SHELF LIFE STUDIES WITH MEDIA DEVELOPED FOR MLM

FREEZE DRIED MEDIA WAS STORED AT 4°C FOR 4 MONTHS. FOLLOWING STORAGE, DUPLICATE ALIQUOTS OF EACH BROTH WERE REHYDRATED WITH DISTILLED WATER. TESTING FOR SELECTIVITY WAS AS FOLLOWS: ONE REHYDRATED ALIQUOT WAS TESTED AGAINST ITS SPECIFIC ORGANISM; THE OTHER ALIQUOT WAS INOCULATED WITH A MIXTURE OF ORGANISMS. OBSERVATIONS WERE MADE FOLLOWING INCUBATION FOR 16 HOURS AT 35°C.

BROTH	SPECIFIC ORGANISM USED	RESULTS (16 HR)	RESULTS OF MIXTURE CHALLENGE	ORGANISMS IN MIXTURE
COLIFORM	ESCHERICHIA COLI	+	0	MIMAPOLYMORPHA HERELLEA VAGINICOLA
*CETRIMIDE	PSEUDOMONAS AERUGINOSA	+	0	ALPHA STREPTOCOCCUS MICROCOCCUS RUBENS
*UREASE	PROTEUS MIRABILIS	+	±	SARCINA LUTEA GAFFKYA TETRAGENA
SALMONELLA	SALMONELLA PARATYPHI B	+	0	BACILLUS SUBTILIS SERRATIA MARCESCENS
*STREPTOCOCCUS	BETA STREPTOCOCCUS	+	0	FLAVOBACTERIUM BREVE NOCARDIA CORALLINA
STAPHYLOCOCCUS	STAPHYLOCOCCUS AUREUS	+	± (S ALBUS)	BACTERIUM ANITRATUM ACHROMOBACTER ANITRATA
MYCOSEL	CANDIDA ALBICANS	+	0	ALCALIGENES FAECALIS STAPHYLOCOCCUS ALBUS
SYNTHETIC FUNGI	ASPERGILLUS NIGER	+	0	

+ = GROWTH
0 = NO GROWTH
* PARALLEL TESTING OF THESE BROTHS STORED 4 MONTHS AT 4°C BUT NOT FREEZE-DRIED SHOWED THAT SELECTIVITY WAS NOT MAINTAINED. (GROWTH OBSERVED IN TUBES INOCULATED WITH VILE MIX).

TABLE 28
SURVIVAL OF ORGANISMS IN RESPECTIVE MLM MEDIUM

ORGANISMS SEEDED INTO APPROPRIATE MEDIA AND INCUBATED 16 HR AFTER ENUMERATION TESTS, MEDIA WERE DIVIDED INTO ALIQUOTS AND STORED FOR SUBSEQUENT SURVIVAL TESTS.

RESULTS

STORAGE IN BROTH 4°C					
ORGANISM	ORGANISMS/ML				
	0 TIME	24 HR	1 WEEK	30 DAYS	60 DAYS
ESCHERICHIA COLI	10^7	10^7	10^7	10^7	10^7
PSEUDOMONAS AERUGINOSA	10^7	10^7	10^7	10^7	10^7
PROTEUS MIRABILIS	10^6	10^6	10^2	<10	0
S. TYPHOSA	10^6	10^6	10^3	<100	0
BETA STREPTOCOCCUS	10^7	10^7	10^7	10^5	<10
STAPHYLOCOCCUS AUREUS	10^7	10^7	10^7	10^7	10^7
CANDIDA ALBICANS	10^7	10^7	10^7	10^7	10^7
HERELLEA VAGINICOLA	10^6	10^6	10^6	10^6	10^6
ASPERGILLUS NIGER	10^7	10^7	10^7	10^5	<10
STORAGE IN BROTH −79°C					
ORGANISM	ORGANISMS/ML				
	0 TIME	24 HR	1 WEEK	30 DAYS	60 DAYS
ESCHERICHIA COLI	10^7	10^7	10^7	10^7	10^6
PSEUDOMONAS AERUGINOSA	10^7	10^6	10^6	10^5	10^5
PROTEUS MIRABILIS	10^6	10^6	10^5	10^5	0
S. TYPHOSA	10^6	10^5	10^4	10^4	0
BETA STREPTOCOCCUS	10^7	10^5	10^5	10^4	10^3
STAPHYLOCOCCUS AUREUS	10^7	10^7	10^7	10^7	10^7
CANDIDA ALBICANS	10^7	10^7	10^7	10^7	10^7
HERELLEA VAGINICOLA	10^6	10^6	10^6	10^6	10^4
ASPERGILLUS NIGER	10^7	10^7	10^7	10^7	10^3

UREASE MEDIUM (PROTEUS)

Glucose	1g/L
Gelysate	2g/L
Urea	30g/L
KH_2PO_4	1.4g/L
K_2HPO_4	1.0g/L
NaCl	5g/L
$MgSO_4$	10g/L

Adjust pH to 6.8 with phosphates. Filter sterilize.

SYNTHETIC BROTH FOR FUNGI

Sucrose	30g/L
$NaNO_3$	3g/L
$MgSO_4$	0.5g/L
KCl	0.5g/L
Na_2HPO_4	1.2g/L
KH_2PO_4	0.05g/L
Chloramphenicol	0.05g/L
Phenol Red	.025g/L

Dissolve - no heat - adjust pH to 7.3 with phosphates. Filter Sterilize. Add 25.0 mg/L Colymycin and 25.0 mg/L Naladixic acid.

MYCOSEL BROTH (CANDIDA)

Phytone peptone	10g/L
Dextrose	10g/L
Cyclohexamide	0.4g/L
Chloramphenicol	0.05g/L

Heat slightly to dissolve. Adjust pH to 6.9 with phosphate. Filter Sterilize. Add 25.0 mg/L Colymycin and 25.0 mg/L Naladixic Acid.

CETRIMIDE BROTH (PSEUDOMONAS)

BioCert. Tryptic Soy Broth	30g/L
Cetrimide	0.2g/L

Heat slightly to dissolve. Adjust pH to 7.5 with phosphates. Filter Sterilize.

COLIFORM BROTH

Lactose	10g/L
Gelysate	10g/L
Sodium desoxycholate	10g/L
Brilliant green	13.3 mg/L

Dissolve lactose and gelysate HCl or NaOH to pH 7.4. Add desoxycholate. Do not boil. Filter sterilize. Add Brilliant Green.

SALMONELLA BROTH

Sodium Acid Selenite	5g/L
L-lysine MonoHCl	10g/L
Ammonium Chloride	3g/L
Yeast extract	1g/L
KH_2PO_4	1.8g/L
Phenol Red	0.03g/L

Dissolve no heat - Adjust pH to 6.7 with phosphates. Filter sterilize.

SALICIN NEOMYCIN BROTH (STREPTOCOCCUS)

The concentrations of Neomycin Sulfate and KTe have been increased in this revised formula.

In distilled H_2O, 05 ml, add:

Trypticase	1.5g
Phytone	0.5g
NaCl	0.5g
Salicin	1.0g

Heat to dissolve. Adjust pH to 7.3 with phosphates. Filter sterilize. When cool, add 0.5 ml KTe (1% stock solution). 5 ml stock neomycin-sulfate (.5 mg in 100 ml H_2O) yields a 0.025 mg% final medium concentration.

NEISSERIA BROTH

Trypticase	0.75g
Thiotone	0.75g
K_2HPO_4	0.4g
KH_2PO_4	0.1gm
NaCl	0.5g
Water	85.0 ml

Autoclave above

Aseptically add 0.25g hemoglobin in 10 ml sterile water

1.0 ml supplement B

1.0 ml Colymycin stock = 2500 mcg/100 ml final

1.0 ml Vancomycin stock = 3000 mcg/100ml final

1.0 ml Polymycin stock = 1500 mcg/100 ml final

1.0 ml Nystatin = 3750 units/100 ml final

pH 7.2

HERELLEA BROTH

1g Ethanol

0.3g NH_4Cl

0.5g NaCl

0.1 g K_2HPO_4

0.01 g $MgSO_4$

99ml H_2O

pH 6.0

0.1 ml Nystatin (5000 units)

MIMA BROTH

4% Tween 40

.006 Nile Blue A

4% NaCl

.05% Yeast extract

0.3% K_2HPO_4

pH 8.9

0.5% NH_4Cl

4.0 DISCUSSION

Our MDAC-East bioengineering and microbiology team is proud of an on-time successful contract completion. In one year, our MLM development team has progressed from a proven feasibility concept to a diagnostic tool proven capable of accepting human throat, urine, and fecal clinical specimens.

The MLM electronics prove to be sensitive and reliable. The sequential sampling MLM developed during this contract has the versatility to detect growth in a miniaturized detection cell array.

Clinical evaluation has demonstrated that the developed MLM culture media compare favorably with standard clinical culture media.

Although electronic refinements and selective media were important, the most unique and important contract development is the filter cassettes.

The filter cassette permits the clinical specimen acceptance, from clear liquids to semisolid material. Serial filter beds not only dilute the specimen but also clarify dense materials such as feces. The freeze-dried use to pre-program the cassette extends the media shelf life and allows the using of a single cassette diluent.

Another important study aspect is the discovery that certain selective media, in conjunction with the MLM, affect characteristic specific microorganism growth curves. Examples of this phenomenon are the hump in Pseudomonas growth curve and the curve regression obtained with Proteus species. Other workers have not previously reported these events. Our technique of rapid specific organism detection by noting characteristic growth curves in mixed bacterial cultures is a significant tool opening new microbial detection vistas. Our technique also explains how not totally selective media can still be MLM useful.

The interpretation of obtained MLM data is complex enough to justify a diagram and explanation. Therefore, to clarify the many concepts which combine to provide detection of microorganisms by the MLM, we present a hypothetical example below. In this example, a throat swab is assumed to have been made. To illustrate, assume the throat swab has been diluted in the water diluent provided in the cartridge and the filter cassettes have been filled with this inoculum. The cassette has been preprogrammed with preserved selective media

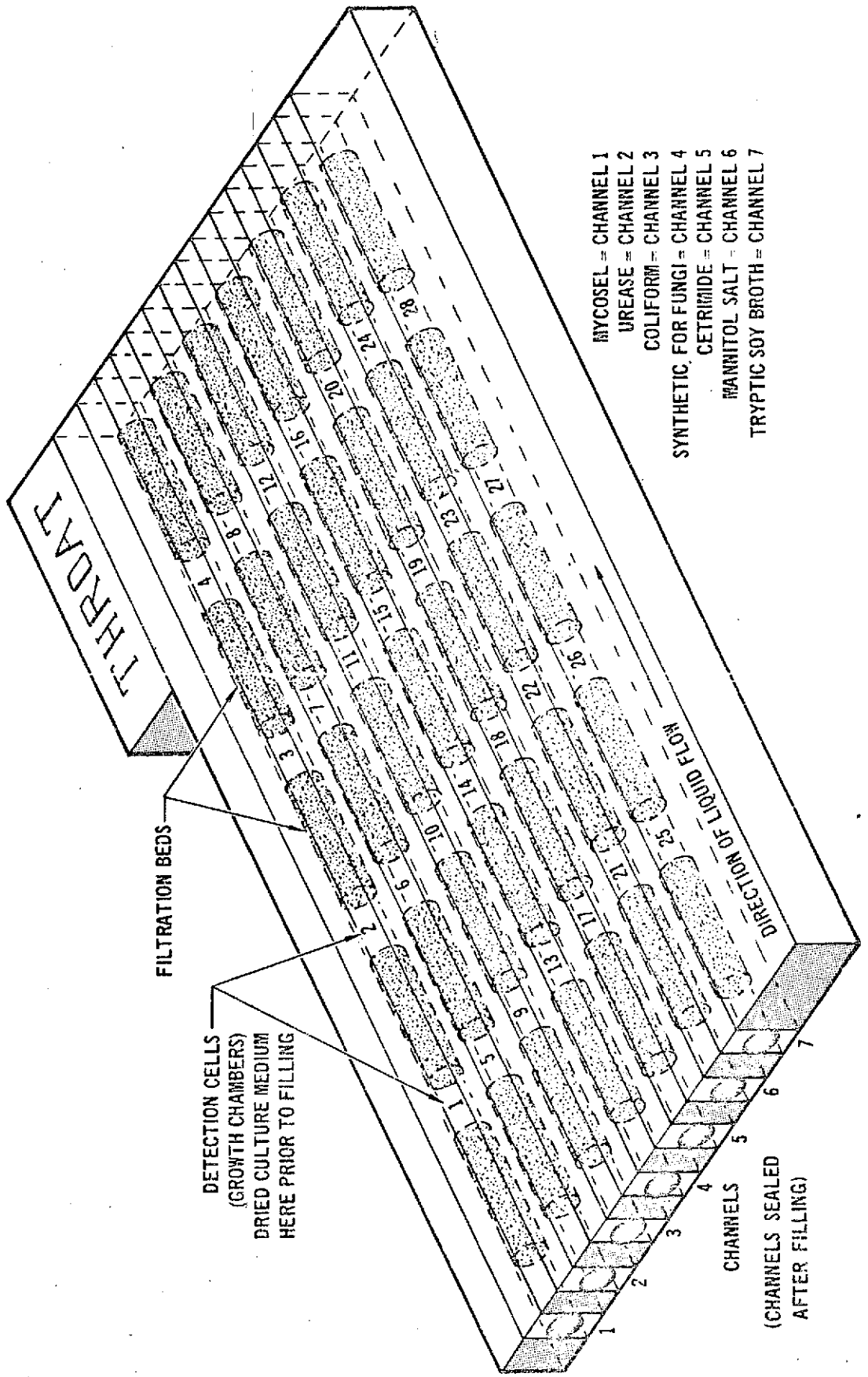
as depicted in Figure 56. (The figure is conceptual. Due to machining problems the channels are not straight, but zigzag, and one channel represents one cassette. However, the principal is the same.) The cassette is then put into the MLM for analysis. At the end of 16 hours the MLM has detected the following.

Channel 1, cells 1, 2, 3, 4 all negative
Channel 2, cell number 5 half scale deflection, cell 6 full scale deflection, cells 7 and 8 negative
Channel 3, cell 9 one-quarter scale deflection, cells 10, 11, and 12 negative
Channel 4, cells 13, 14, 15, and 16 negative
Channel 5, cells 17, 18, 19, and 20 negative
Channel 6, cells 21, 22 full scale deflection, cell 23 half scale deflection and cell 24 negative
Channel 7, cells 25, 26, 27 full scale deflection, cell 28 half scale deflection

Conclusions based on the MLM analysis are as follows:

1. Total mesophilic aerobes present exceed 1×10^4/ml original sample Determination of this information is possible because filtration and dilution has established most probable number from channel 7.
2. No fungi have been detected.
3. Proteus species is present and total numbers exceed 1×10^2, but are less than 1×10^3. Proteus species is most probably present because the half scale reading in cell five, coupled with a full scale reading in cell six, indicates proteus-type urease activity which first swings full scale, then backs off to half scale before swinging back again.
4. Coliforms could be present in small numbers; however, the presence of large Proteus numbers would also give a slight growth in this channel. It is therefore most probably Proteus.
5. Pseudomonas is not present in sufficient numbers for detection.
6. Staphylococcus aureus is probably present and total number/ml exceeds 1×10^3. It is not possible to rule out the presence of Staphylococcus epidermidis which may be overgrown by S. aureus.

FIGURE 56
DIAGRAM OF RIGID SAMPLE HOLDER WITH SEVEN CHANNELS CONTAINING FREEZE DRIED MEDIA AND FILTRATION BEDS



In our example, the detection cell numbers, fluid channels, and media type were arbitrarily chosen for illustrative purposes. It is obvious that other examples of preprogrammed channels and media could be substituted.

In actual practice we discovered that preprogramming the cassette is very important. The freeze-dried media amount and location is critical, and differs for each medium. We empirically determined that optimum amount and placement formula was 50 percent of total weight placed prior to first detection cell, 25 percent prior to second detection cell, and 12.5 percent in the remaining detection cells. With the rapidly soluble medium for Pseudomonas, 90% of the medium must precede the first detection cell. Growth curve results obtained with improper media placement usually show illogical growth sequences; with the most dilute inoculum growing faster than the preceding growth chamber containing one order of magnitude more organisms. This sequence is due to insufficient growth chamber nutrients.

Defining the problem, assume each growth chamber is preprogrammed with equal dried culture medium amounts. During rehydration, the fluid flowing through the first chamber dissolves some of the medium and carries it along to the next chamber and so on. It is obvious that such a system will have different medium concentrations in each growth chamber. To avoid such undesirable differences it was necessary to place decreasing dried medium amounts in subsequent growth chambers. We determined necessary amounts by trial and error, using densitometer measurements as a comparison basis for each rehydrated chamber.

Two media required the addition of ingredients which cannot be freeze-dried. These were media for beta hemolytic streptococci which requires red blood cells, and herellea broth requiring ethanol. The blood cell requirement is a sticky problem. Preserved but undried red blood cells can be used for time periods up to six weeks, and therefore can be used with special diluent. However, every effort was made to rehydrate treated red blood cells. At contract's end, this effort's success was uncertain due to equal failure numbers during process repeat attempts. Adding dry sodium ethoxide to the heavily buffered herellea medium can substitute for ethanol, but shelf life is unknown. In either case, if substitute media cannot be developed, both can be used as special case media requiring adding wet media instead of dry.

We developed other quite reliable media during the program. Our greatest confidence is detecting Staphylococcus aureus; Pseudomonas aeruginosa; Streptococcus pyogenes, type A beta hemolytic; Proteus species; Coliforms; Salmonella species, especially typhosa; paratyphi A and B; and Candida albicans.

We can detect Neisseria meningitidis in Proteus' absence, but we cannot distinguish it from Proteus species in the MLM. However, our MLM can detect the peculiar proteus urease activity, and we assume that a positive reaction with the Neisseria medium in the absence of Proteus means N. meningitidis is present.

Diplococcus pneumoniae detection is possible only when the number of D. pneumoniae in relation to other organisms is quite high - approximately 1×10^3 diplococci are required. The medium is effective because the other organisms are retarded, and if organism inoculum size, other than D. pneumoniae, is relatively large - D. pneumoniae cannot be detected.

Our Mima and Herellea media are effective for the strains tested; however, due to confusion as to what is a Herellea or Mima, the media are questionable. If the question is "Mima or Herellea?" then our confidence level is quite high.

We should also point out that a cassette can be programmed with an all purpose media, such as tryptic soy broth to permit a rough order of magnitude determinant of total organisms present. Such an approach even has clinical applications, as with urinary tract infections where the presence of 1×10^4 organisms is significant, regardless of species.

Our MLM sensitivity is quite good and illustrated by the fact that the MLM detected Candida albicans in a throat swab which standard culture techniques had missed. Careful repeat testing with standard culture techniques confirmed the MLM detection.

5.0 CONCLUSIONS

Our prime conclusion is that the MLM can function as a diagnostic instrument to detect medically important organisms in human throat, skin, urine, and fecal clinical specimens.

Other important conclusions are as follows:

1. We developed MLM-compatible selective media for all organisms and groups listed in the contract Statement of Work.
2. Most of the media developed for MLM use can be freeze-dried for preprogramming the rigid sample holder detection chambers. Two cannot be freeze-dried but are highly effective and useful.
3. Our sequential filtration beds concept yields data useful for determining relative organism numbers present in clinical specimens.
4. We have proven feasible the concept of one sample or specimen diluent for all fluid channels.
5. Our developed MLM media have selectivity for freshly isolated microorganisms as well as laboratory strains.
6. Our MLM media select for microorganisms in the presence of human throat, urine, and fecal clinical materials.
7. Minor modifications of the MLM electronics have been made which accommodate the evolved rigid sample holder, now called Filter Cassette.
8. The present requirement for MLM operator attention and control is greatly simplified by the new electronic modification called the Sequential Sampling MLM (SSAMM) system. Our test results proved highly successful and have provided several design inputs for an expanded system capable of viewing a detection cell array.

6.0 RECOMMENDATIONS

We developed the MLM into an important diagnostic instrument. Although designed to operate under null gravity conditions, its usefulness in medical or industrial laboratories is obvious. The MLM is in its infancy, but its potential is unlimited. The instrument as now developed, has successfully detected medically important organisms in human clinical specimens. It can detect and determine most probable numbers in a few hours. No other instrument known is capable of that combination of abilities.

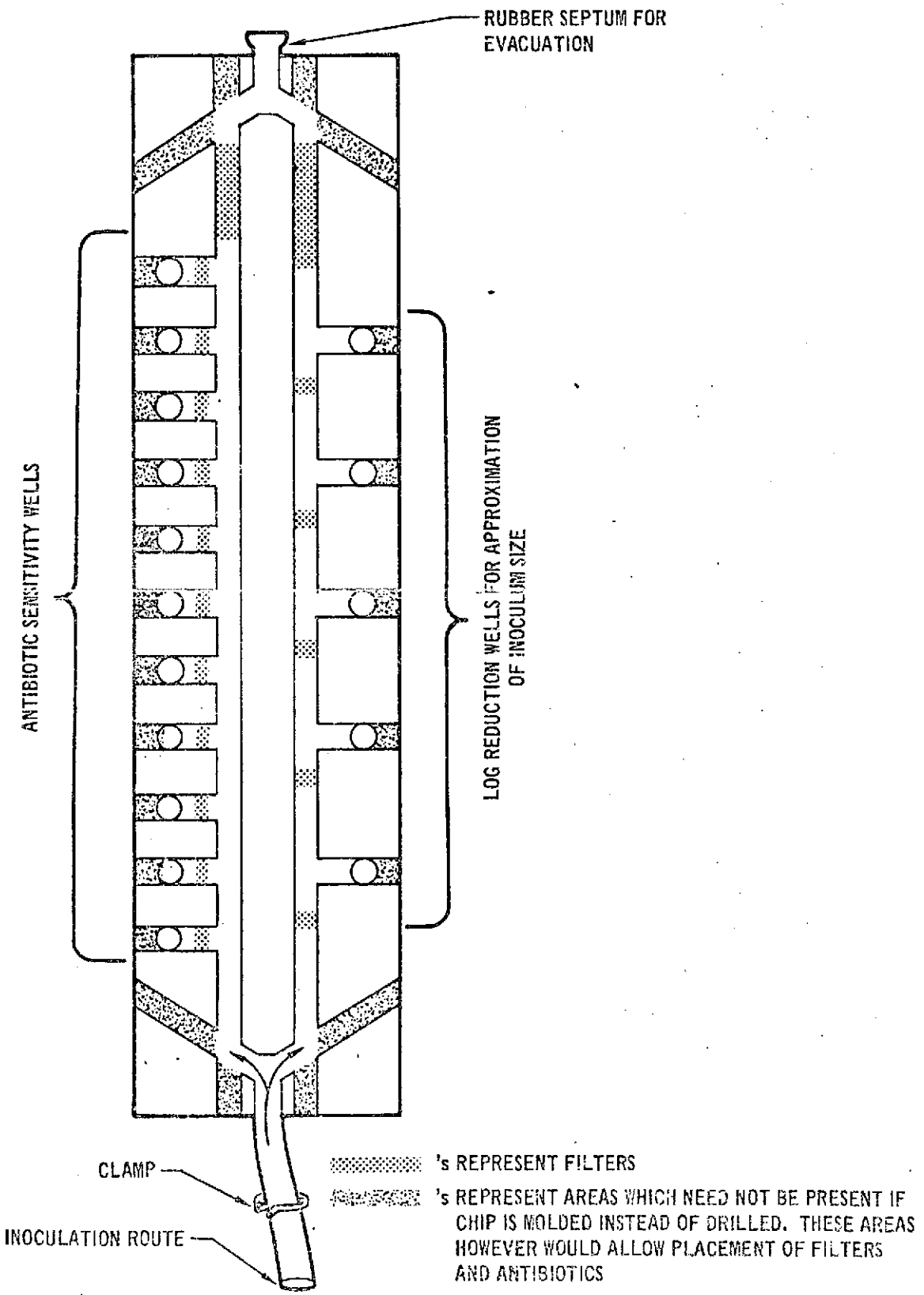
It is therefore recommended that the development of this instrument be continued to include other medically important organisms and to include the development of the capability to automatically determine drug sensitivity.

Some design work along these lines has been initiated. Figure 57 presents one approach to a filter cassette capable of drug sensitivity determination which is MLM compatible.

If the instrument is to become a flight item there is much work to be done, but there is no doubt that it will function as planned.

FIGURE 57

DESIGN OF CASSETTE CAPABLE OF DETERMINING ANTIBIOTIC SENSITIVITY OF MICROORGANISMS



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